Aerobic function and skeletal muscle plasticity in health and disease

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Flurin Item

von Marmorera (GR)

Promotionskomitee

Dr. Marco Toigo (Leitung der Dissertation)

Prof. Dr. Roland H. Wenger (Vorsitz)

Prof. Dr. med. Urs Boutellier

Prof. Dr. Christoph Handschin

Zürich, 2011

This work has been performed under the supervision of

Dr. Marco Toigo and

Prof. Dr. Roland H. Wenger

at the Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, and at the Department of Human Movement Science, ETH Zurich, 8057 Zurich, Switzerland

Summary

In recent decades, chronic diseases have reached epidemic proportions in the industrialised world. A physically inactive lifestyle has been identified as a major factor causing maladaptations leading to coronary heart disease, stroke, hypertension, type 2 diabetes, osteoporosis, breast cancer, and colon cancer. Regular physical activity prevents the development of obesity and induces a multitude of favourable adaptations within skeletal muscle and the cardio-respiratory system, which have positive outcomes for both the prevention and treatment of metabolic diseases. Specifically, high aerobic function is related to physical activity whereas low aerobic function is related to physical inactivity and various metabolic diseases. In order to gain a better understanding of the mechanisms underlying aerobic function and skeletal muscle plasticity it is fundamental to identify which specific exercise stimuli leads to what molecular response, and how this molecular response relates to the structural, contractile, and metabolic adaptation. Thus, the present thesis was aimed at providing new insights into the mechanisms underlying aerobic function and skeletal muscle plasticity by using an integrative approach including cell culture, rodent, and human models.

In a first study, in order to shed light upon the relationship of aerobic function and type 1 diabetes, we investigated factors that may limit oxidative capacity and aerobic exercise performance in young untrained women with type 1 diabetes. Calf muscle oxidative capacity was not different between untrained women with type 1 diabetes and healthy women of similar age and activity levels. Notably, HbA1c was negatively correlated with mitochondrial capacity in women with type 1 diabetes. Although HbA1c was negatively correlated with cardiac output in women with type 1 diabetes, maximal oxygen consumption, cardiac output, endurance capacity, skeletal muscle

oxidative enzyme activity, and capillary-to-fibre ratio were not reduced in women with type 1 diabetes compared to healthy women. These results indicate that oxidative capacity depends on HbA1c in untrained women with type 1 diabetes but aerobic function is not reduced relative to untrained healthy women. Thus, it is important to accurately control glycaemic status in future studies investigating the aerobic function in patients with type 1 diabetes.

In a second study, we aimed at establishing a cell culture model to closely recapitulate the plastic changes in gene expression as observed in aerobically trained skeletal muscles of mice. In electrically stimulated C2C12 mouse muscle cells the transcriptional adaptations were almost identical to those in endurance trained skeletal muscles of mice, but differed from the acute effects of exercise on muscle gene expression. In addition, significant alterations in the expression of myofibrillar proteins indicated that this *in vitro* exercise model could be used to modulate the fibre-type of muscle cells in culture. Our data thus describe an experimental cell culture model for the study of at least some of the transcriptional aspects of skeletal muscle adaptation to physical activity.

Typically, aerobic function can be improved by repeated endurance exercise but not by resistance exercise. To overcome the specificity of this adaptive response we aimed in a third study at designing a new resistance exercise model with superimposed stimuli to induce endurance type adaptations and to test its effectiveness in humans. Indeed, 5 weeks of whole-body vibration training with superimposed heavy resistance exercise and sustained vascular occlusion (VRO) increased capillary-to-fibre ratio, skeletal muscle oxidative enzyme activity, myosin heavy chain type 1 fibre proportion, and endurance capacity. In a fourth study, we analysed the molecular bases underlying these adaptations. A single bout of VRO increased the expression of vascular endothelial growth factor (VEGF) mRNA through reactive oxygen species (ROS)-activated peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) probably in a hypoxia-inducible factor 1α (HIF-1α) independent manner. In conclusion, modified high-intensity resistance exercise activates gene programmes typically linked to endurance exercise.

Zusammenfassung

In den letzten Jahrzehnten haben chronische Krankheiten in der industrialisierten Welt epidemieartig zugenommen. Ein körperlich inaktiver Lebensstil gilt als Hauptursache für viele verschiedene Krankheiten wie koronare Herzkrankheit, Schlaganfall, Bluthochdruck, Typ 2 Diabetes, Osteoporose, Brust- und Darmkrebs. Regelmässige körperliche Aktivität kann die Entstehung von Fettleibigkeit verhindern und zu vielen Anpassungen in der Skelettmuskulatur und im Herzkreislaufsystem führen und dadurch Stoffwechselerkrankungen entgegenwirken. Insbesondere hängt eine gute aerobe Muskelfunktion mit körperlicher Aktivität zusammen, währenddem eine verminderte aerobe Muskelfunktion mit körperlicher Inaktivität und vielen Stoffwechselerkrankungen in Zusammenhang gebracht wird. Um ein besseres Verständnis der Mechanismen der aeroben Muskelfunktion und -plastizität zu erhalten, ist es notwendig zu verstehen, welche spezifischen Trainingsreize zu welcher molekularen Antwort führen und welche molekulare Antworten welche strukturellen, kontraktilen und metabolischen Adaptationen verursachen. Aus diesem Grund bestand das Ziel der vorliegenden Arbeit darin, neue Erkenntnisse über die aerobe Muskelfunktion und -plastizität zu erlangen. Zu diesem Zweck haben wir in unseren Studien einen integrativen Ansatz gewählt und dabei Zellkultur-, Nagetier- und Humanmodelle angewendet.

In der ersten Studie untersuchten wir den Zusammenhang zwischen aerober Muskelfunktion und Typ 1 Diabetes. Wir haben Faktoren, welche die oxidative Kapazität und aerobe Leistungsfähigkeit determinieren, in untrainierten jungen Frauen mit Typ 1 Diabetes gemessen und mit gesunden untrainierten Frauen im gleichen Alter verglichen. Die oxidative Kapazität im Unterschenkelmuskel hat sich zwischen untrainierten jungen Frauen mit Typ 1 Diabetes und gesunden untrainierten Frauen im

gleichen Alter nicht unterschieden. Bemerkenswert ist, dass bei den Typ 1 Diabetikerinnen das HbA1c negativ mit der mitochondrialen Kapazität korrelierte. Obwohl bei den Typ 1 Diabetikerinnen das HbA1c auch mit dem Herzminutenvolumen negativ korrelierte, waren die maximale Sauerstoffaufnahme, Herzminutenvolumen, Ausdauerleistungsfähigkeit, Aktivität von oxidativen Enzymen in der Skelettmuskulatur und das Verhältnis der Kapillaren zu den Skelettmuskelfasern, im Vergleich mit gleichaltrigen gesunden Frauen, nicht vermindert. Diese Ergebnisse zeigen auf, dass bei untrainierten Frauen mit Typ 1 Diabetes die oxidative Kapazität vom HbA1c abhängig ist, die aerobe Muskelfunktion verglichen mit gesunden untrainierten Frauen aber nicht vermindert ist. Daher ist es wichtig, in zukünftigen Studien das HbA1c sorgfältig zu kontrollieren, wenn man die aerobe Muskelfunktion untersuchen will.

In einer zweiten Studie entwickelten wir ein Zellkulturmodell, in welchem wir die gleichen Genexpressionsveränderungen beobachten konnten, wie in Skelettmuskeln von ausdauertrainierten Mäusen. In elektrisch stimulierten C2C12 Mausmuskelzellen waren die transkriptionellen Adaptationen beinahe identisch mit denen in Skelettmuskeln von ausdauertrainierten Mäusen. Sie unterschieden sich aber von den akuten transkriptionellen Antworten in Skelettmuskeln von Mäusen. Zudem zeigten sich signifikante Veränderungen in der Expression von myofibrillären Proteinen. Unsere Studienergebnisse zeigen, dass wir dieses Zellkulturmodell dafür verwenden können, um transkriptionelle Veränderungen im Skelettmuskel nach körperlicher Aktivität und Veränderungen des Skelettmuskelfasertypus zu untersuchen.

Üblicherweise kann die aerobe Muskelfunktion mit regelmässigem Ausdauertraining nicht aber mit Krafttraining verbessert werden. Um diese spezifischen Anpassungen zu umgehen, haben wir in einer dritten Studie ein neuartiges Trainingsmodell entwickelt. Unser Ziel war es, mit einem modifizierten Krafttraining Adaptionen hervorzurufen, welche normalerweise mit Ausdauertraining einhergehen. Das modifizierte Krafttraining bestand aus einer Kombination aus intensivem Vibrations- und Krafttraining mit gleichzeitig unterbundenem Blutfluss zu und aus den Beinen. Wir konnten zeigen, dass fünf Wochen modifiziertes Krafttraining zu einem erhöhten Verhältnis der Kapillaren zu den Skelettmuskelfasern führte und dies mit einer erhöhten Aktivität von oxidativen Enzymen in den Skelettmuskeln einherging. Zudem nahm der Anteil der Typ I Muskelfasern und die Ausdauerleistungsfähigkeit zu.

In der vierten Studie untersuchten wir die molekularen Mechanismen, welche zu den beschriebenen Adaptationen führten. Ein einzelnes modifiziertes Krafttraining erhöhte die mRNA Expression von “vascular endothelial growth factor” (VEGF) und “peroxisome proliferator-activated receptor γ coactivator 1α” (PGC-1α). Möglicherweise aktivierten “reactive oxygen species” (ROS) flussabwärts PGC-1α, welches weiter VEGF auf eine “hypoxia-inducible factor 1α” (HIF-1α) unabhängige Weise induzierte. Unsere Studienergebnisse belegen, dass ein modifiziertes hochintensives Krafttraining Genprogramme induzieren kann, welche üblicherweise nach Ausdauertraining beobachtet werden.

Contents

Abbreviations

AMPK	AMP-activated protein kinase
ATPSyn	ATP synthase subunit
CLT	Constant-load cycling exercise test
Cn	Calcineurin
COX5	Cytochrome c oxidase subunit 5b
Cpt1b	Carnitine-palmitoyltransferase 1b
Cyt c	Cytochrome c oxidase
4E-BP1	4E-binding protein 1
eIF	Eukaryotic initiation factor
EMG	Electromyography
EPS	Electric pulse stimulation
ERRα	Estrogen-related receptor α
GABPA	GA-binding protein A
Glut	Glucose transporter
GSK3β	Glycogen synthase kinase 3β
GXT	Graded cycling exercise test
GYS1	Glycogen synthase 1
HbA1c	Glycosylated haemoglobin A1c
HDAC	Histone deacetylase
HIF-1	Hypoxia-inducible factor
HKII	Hexokinase II
HOMA-IR	Homeostasis model assessment-Insulin resisance
IRS	Insulin receptor substrate
k_{PCr}	Phosphocreatine recovery rate
LDH	Lactate dehydrogenase

mATPase	Myofibrillar adenosinetriphosphatase
MCAD	Medium chain acyl-CoA dehydrogenase
MEF2	Myocyte enhancer factor 2
MnSOD	Manganese superoxide dismutase
mTOR	Mammalian target of rapamycin
MVF_P	Maximal force acting on the pedal
MYH	Myosin heavy chain
MyHC	Myosin heavy chain
Ndufb5	NADH-ubiquinone oxidoreductase 1β subcomplex 5
NFAT	Nuclear factor of activated T-cells
NRF	Nuclear respiratory factor
OXPHOS	Genes encoding oxidative phosphorylation proteins
PCr	Phosphocreatine
PDH	Pyruvate dehydrogenase
PFKm	Phospho fructokinase, muscle type
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1α
PHD3	Prolyl hydroxylase dehydrogenase 3
P_i	Inorganic phosphate
PI3K	Phosphatidylinositol-3 kinase
PKD1	Protein kinase D1
^{31}P-MRS	^{31}Phospho-magnetic resonance spectroscopy
PPARγ	Peroxisome proliferator-activated receptor γ
Rheb	Rat homologue enriched in brain
ROS	Reactive oxygen species
S6K1	Ribosomal protein S6 kinase
TBP	TATA box binding protein
Tfam	Mitochondrial transcription factor A
TSC	Tuberous sclerosis complex
VEGF	Vascular endothelial growth factor
VRO	Vibration exercise + resistance exercise + vascular occlusion
XD	Xanthine dehydrogenase
XO	Xanthine oxidase

Chapter 1

Introduction

In recent decades, chronic diseases have reached epidemic proportions in the industrialised world (Booth et al. 2002). A physically inactive lifestyle has been identified as a major factor causing maladaptations leading to chronic diseases (Booth et al. 2008, Booth and Lees 2007, Centers for Disease Control and Prevention 2006). Physical inactivity increases the risk of coronary heart disease, stroke, hypertension, type 2 diabetes, osteoporosis, breast cancer, and colon cancer by between 30% and 60% (Booth and Laye 2009, Katzmarzyk and Janssen 2004, Fig. 1.1). Regular physical activity (together with a balanced energy intake) prevents the development of obesity and induces a multitude of favourable adaptations within skeletal muscle and the cardio-respiratory system, which have positive outcomes for both the prevention and treatment of metabolic diseases (Hawley and Holloszy 2009). Booth and Laye (2009) recently pointed out that in order to prove causality between physical inactivity and chronic diseases it is necessary to understand the underlying molecular pathways. However, the mechanisms that mediate the therapeutic effects of exercise and the pathological changes elicited by a sedentary lifestyle remain enigmatic and have to be fully elucidated (Handschin and Spiegelman 2008).

Skeletal muscle plasticity. The human body contains about 660 different skeletal muscles accounting for about 45% of body mass (Brooks et al. 2005). Beside the primary purpose as a motor for locomotion, skeletal muscle is an important tissue for whole-body energy metabolism and substrate turnover, and therefore makes a

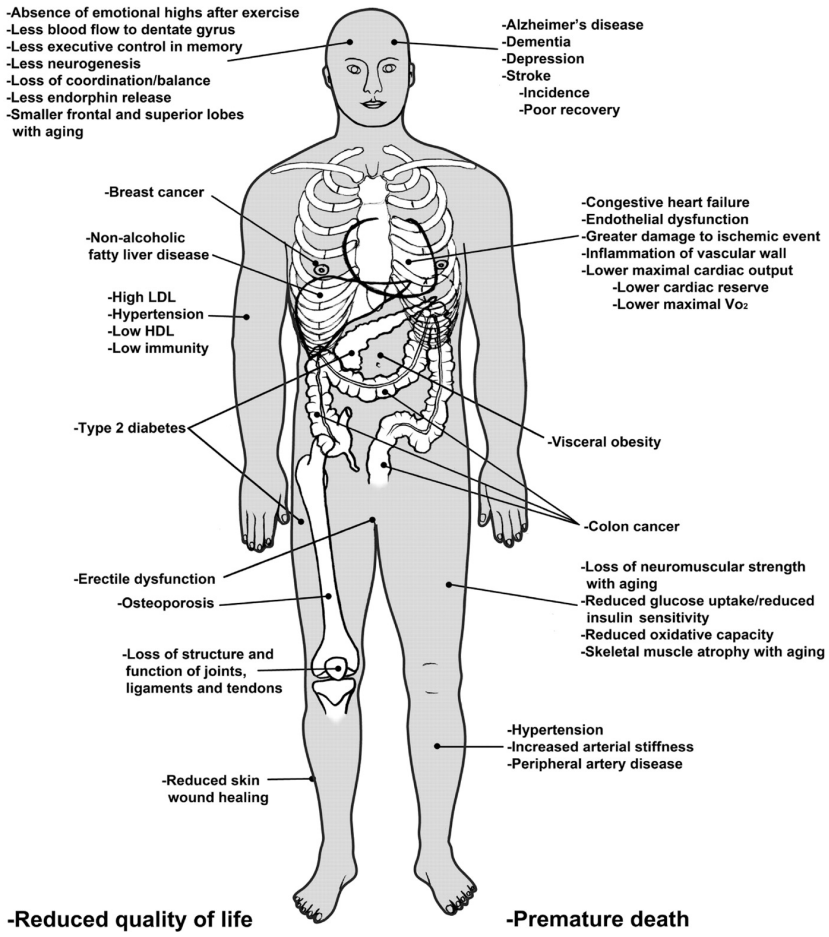

Figure 1.1: Chronic health diseases induced upon decreasing physical activity from high to low levels (Booth and Laye 2009).

major impact on metabolic health. Furthermore, skeletal muscle is a highly malleable tissue (Baldwin and Haddad 2002, Flueck 2006, Flueck and Hoppeler 2003, Schiaffino et al. 2007) whose structure and function can be changed as a consequence of contractile activity, substrate supply, environmental factors such as hypoxia and thermal stress, and ageing (Flueck and Hoppeler 2003). Skeletal muscle plasticity has been defined as "the ability of a given muscle cell to alter 1) the quantity (amount) of and/or 2) the type of protein (*i.e.* phenotype or isoform) comprising its different subcellular components in response to any type of stimulus

that disrupts its normal homeostasis" (Booth and Baldwin 1995). In detail, the specific stimuli perturb the skeletal muscle's homeostasis, and this signal is in turn mechano-chemically transduced into a molecular and cellular response (Toigo and Boutellier 2006). As such, the combination of mechanical stress, metabolic stress, and altered calcium flux within skeletal muscle determines the mechano-chemical transduction, based on the individuals' genetic background, age, gender, and several other factors. These molecular and cellular responses finally lead to specific structural and metabolic adaptations that result in task-specific functional enhancements or clinical effects (*i.e.* adaptational effects) (Fig. 1.2; Toigo and Boutellier 2006).

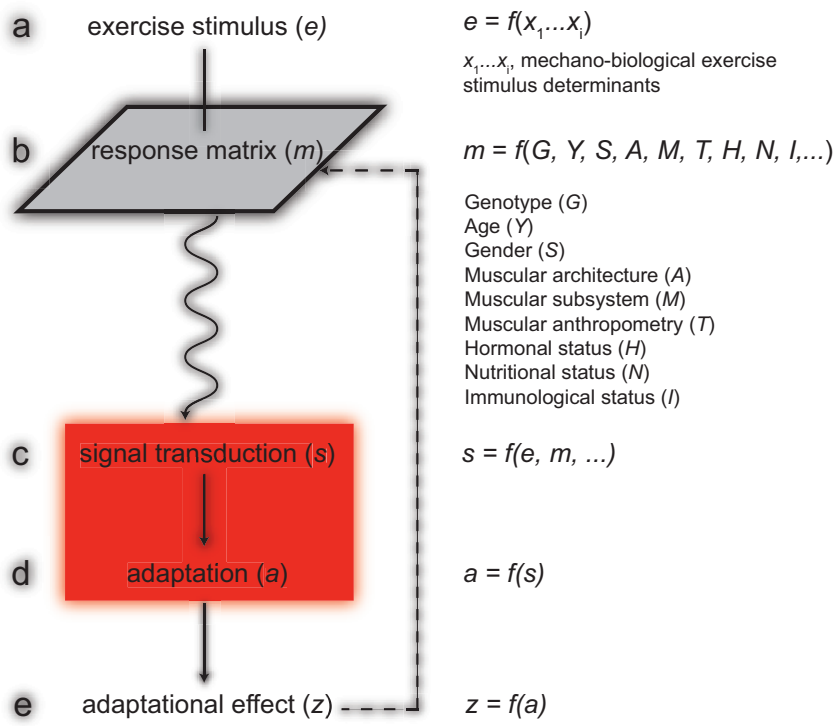

Figure 1.2: Model of the signalling transduction pathways induced by specific exercise stimuli which lead to a structural adaptation with associated adaptational effect (Toigo and Boutellier 2006). An exercise stimulus with specific mechano-biological characteristics (a) induces a specific signal transduction pathway (c) based on the respective response matrix (b) which leads into a quantitative and/or qualitative adaptation (d). Such an adaptation can finally lead to an adaptational effect (e). Of important note is, that there exist only a causal connection between (c) and (d) (*red shading*).

Skeletal muscle function

Excitation-contraction coupling. The contraction of the mammalian skeletal muscle is neurally controlled. The activity of an efferent α-motor neuron induces an action potential at the neuromuscular junctions. This action potential triggers the release of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasmic space. Subsequently, Ca^{2+} binds to troponin provoking an ATP-dependent interaction changes of myosin and actin in the contractile apparatus (excitation-contraction coupling) (Huxley 1988). This force production is energy-dependent, thus ATP gets hydrolysed to ADP, inorganic phosphate, and free energy. There are several systems, which cope with the increasing energy demand during the excitation-contraction coupling. The first system is composed of high-energy phosphate transformations involving the coupling of the *creatine kinase* and *adenylate kinase* enzyme systems. Creatine phosphate provides a reserve of phosphate energy to regenerate ATP and *adenylate kinase* catalyses the generation of one ATP and one AMP from two ADPs. The second system involves the mobilisation of endogenous glycogen through the glycolytic pathway to regulate the ATP levels independent of oxidative processes. The third system include the regeneration of ATP from ADP through oxidative phosphorylation of reduction equivalents arising from the combustion of carbohydrates and/or fatty acids. Glycolysis yields ATP at high rates but is less efficient in generating ATP yield per mole of glucose than the oxidative phosphorylation process (Booth and Baldwin 1995, Brooks et al. 2005, Flueck and Hoppeler 2003). During continued skeletal muscle activity, the balance between carbohydrate and fat metabolism is controlled by two factors: 1) by the intensity of physical activity and 2) the supply of nutrient from intracellular (intramyocellular lipids, glycogen) and extracellular stores (in liver and adipose tissue). Extracellular substrate delivery depends on the capillary system, which is determined by the conditions for oxygen transfer to skeletal muscle cells (Flueck and Hoppeler 2003).

Skeletal muscle fibres. Skeletal muscle displays a remarkable heterogeneity in terms of fibre type composition. Commonly, skeletal muscle fibres are distinguished either as slow- or fast-twitch. More specifically, skeletal muscle fibres are subdivided based on the expression of the predominant myosin heavy chain (MYH) isoforms, and hybrid fibre types which express a combination of MYH isoforms (Pette and Staron

2000). Basically, mammalian skeletal muscles fibres are classified in terms of their contractile and metabolic properties. Human skeletal muscles express the pure fibre types MYH-1, MYH-2A, and MYH-2X, whereas rodent skeletal muscles additionally express MYH-2B (Tab. 1.1; Smerdu et al. 1994). Skeletal muscles encompass a variety of different fibre types displaying a continuum of pure and hybrid fibre types. The cycling kinetics of the cross-bridge machinery within a muscle fibre is thought to be mediated by the specific activity of its *myosin adenosine triphosphatase* (ATPase) (Caiozzo et al. 1991). Additional qualitative differences exist between fibre types with fibre type specific isoforms of the essential and regulatory myosin light chains, troponin subunits, tropomyosin, α-actinin, and various Ca^{2+}-regulatory proteins (*e.g.* sarcoplasmic reticulum Ca^{2+}-ATPase, calsequestrin, and the α-subunit of the dihydropyridine receptor) (Pette and Staron 2000). There are not only qualitative, but also quantitative differences between fibre types such as metabolic enzyme activity levels, *e.g.* oxidative (*cytochrome c oxidase, succinate dehydrogenase, NADH-tetrazolium reductase*) or glycolytic (*glycogen phos-phorylase*) enzymes. However, quantitative differences do not lead to an exact distinction between fibre types, but rather encompass a continuum with large overlaps in enzyme activity levels between the different MYH isoforms (Pette and Staron 2000). This classification of different MYH-isoforms has been extended to the concept of complex, functional gene groupings within skeletal muscle fibres which adapt independently to environmental stimuli (Spangenburg and Booth 2003). Such gene domains are contractile protein isoforms, mitochondrial volume, myoglobin levels, capillary density, and oxidative enzyme capacity. Contractile activity following neural activation is thought to induce changes in common regulatory factors within the gene domains to modify the muscle fibre phenotype (Spangenburg and Booth 2003).

Exercise. Exercise has been defined in dictionaries as follows: “active: bodily exertion for the sake of restoring the organs and functions to a healthy state or keeping them healthy” or as “regular or repeated use of a faculty or bodily organ or bodily exertion for the sake of developing and maintaining physical fitness” (Booth and Thomason 1991). It has been suggested that these definitions can be applied to human physical activities and to most animal models that mimic human physical activity, involving repeated body exertion invoking multiple organ systems (Booth and Thomason 1991). However, Booth and Thomason (1991) pointed out that interpretations of exercise experiments with animals and muscle cell cultures have

Table 1.1: Characteristics of skeletal muscles in terms of functional units of common metabolic and biochemical properties and their primary adaptive change (Booth and Baldwin 1995).

Properties	Unit types			
	I	IIA	IIX	IIB
Predominant MYH type	I (IIA, IIX)	IIA (I, IIX)	IIX (IIA, IIB)	IIB (IIX)
Contractile speed	Slow (↑)	Fast (↓)	Fast (↑)	Fast (↓)
Myofibril ATPase	Low (↑)	Mod. high (↓)	High (↑)	High (↓)
SR Ca^{2+}-ATPase	Low (↑)	Mod. high (↓)	High	High (↓)
Glycolytic enzymes	Low (↑)	Mod. high (↓)	High (?)	High (↓)
ATP buffering enzyme	Low (↑)	Mod. high (↓)	Mod. high	High (↓)
High-energy phosphate levels	Low (↑)	Mod. (↓)	-	High
Oxidative enzymes	Mod. high (↑)	High (↑)	Mod. high (↑)	Low (↑)
Blood flow	Mod. high	High	-	Low (↑)
Fatigability	Low (↓)	Mod. (↓)	Mod. high (↓)	High (↓)

The myosin heavy chains (MYH) listed in brackets indicate which isoform the primary MYH is transformed into during adaptations in response to either increases or decreases in mechanical activity. The arrowheads listed in brackets indicates either increases or decreases in the relative expression of a given property or enzyme system in response to increases or decreases in mechanical activity imposed on that specific motor unit.

to be done with caution, since these models often do not include the entire body or organ system. Commonly, the term exercise is used to describe a wide variety of (sport) activities. In the traditional view, it is broadly classified as either endurance, resistance (strength), or high-intensity. In order to classify different exercise modalities precisely, two factors have to be considered: 1) load and 2) time duration. Endurance exercise is described as low-load, long duration exercise modality, *e.g.* 40-60 min cycling at a power corresponding to ~65% peak oxygen consumption or as 40-60 min dynamic knee extension exercise at a power corresponding to ~70% of 2 min maximal power. In contrast, resistance exercise is described as high-load, short duration exercise modality, *e.g.* 6-12 repetition with single or multiple sets. High-intensity exercise is defined as the ability to overcome a relatively high load in a very short time period, *e.g.* 4 x 4 min at a power corresponding to ~90-95% peak heart rate followed by 3 min of active resting at 70% peak heart rate or 4-6 repeated

all-out 30 s Wingate tests separated by 4 min recovery. When these described exercise modalities are repeatedly performed for several weeks, the term “exercise training” is used.

Adaptations of skeletal muscle in response to endurance exercise

Repeated bouts of endurance exercise induce mitochondrial biogenesis, oxidative enzyme activity, and angiogenesis, leading to a fatigue-resistant skeletal muscle phenotype and to an increased capacity of skeletal muscle to oxidise fatty acids and carbohydrates (Hood et al. 2006, Holloszy and Booth 1976, Holloszy and Coyle 1984). Moreover, endurance exercise has been shown to promote fibre type transformation from MYH-2B/X to MYH-2A (Fitzsimons et al. 1990). As a consequence of the adaptive increase in mitochondrial mass and oxidative enzyme activity, the disturbance in metabolic homeostasis during submaximal exercise is reduced. This is evidenced by smaller decreases in skeletal muscle ATP, creatine phosphate, and glycogen, and smaller increases in AMP, inorganic phosphate and lactate at a given prolonged submaximal skeletal muscle contractile activity (Constable et al. 1987). Taken together, these adaptations improve the oxidative exercise capacity, making it possible to exercise at higher intensities for longer time periods.

Exercise-induced mitochondrial biogenesis. It has been hypothesised that the adaptation of skeletal muscles following endurance exercise, results from cumulative effects of transient increases in mRNA transcripts encoding specific proteins after consecutive exercise sessions (Flueck and Hoppeler 2003, Pilegaard 2000, Williams and Neufer 1996). The coordination of the gene response to endurance exercise is complex since it has been reported that a single bout of endurance exercise in mice changes the expression of more than 900 genes (Choi et al. 2005). Importantly, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) has been shown to be transiently increased after a single bout of endurance exercise (Baar et al. 2002, Mahoney et al. 2005, Norrbom et al. 2004, Pilegaard et al. 2003, Terada et al. 2002), and to be elevated at the basal level after chronic endurance exercise training (Goto et al. 2000, Russell et al. 2003, Taylor et al. 2005). PGC-1α was originally identified as a coactivator of peroxisome proliferator-activated receptor γ (PPARγ) transcription factor in brown adipose cells (Puigserver et al. 1998).

Subsequent studies revealed that PGC-1α is also expressed in liver and skeletal muscle tissue where it coordinates the expression of both nuclear- and mitochondrial-encoded genes in mitochondrial biogenesis (Puigserver and Spiegelman 2003, Scarpulla 2002). PGC-1α docks on and coactivates the nuclear respiratory factors 1 and 2α (NRF1 and NRF2α) (Scarpulla 2002, Wu et al. 1999). These transcription factors regulate the expression of nuclear genes encoding mitochondrial proteins and induce the expression of mitochondrial transcription factor A (Tfam) (Wu et al. 1999). PGC-1α also activates the transcription factor estrogen-related receptor α (ERRα) (Huss et al., 2004), which elicits a robust induction of vascular endothelial growth factor (VEGF) in cultured muscle cells and mouse skeletal muscle *in vivo* following hypoxia and limb ischaemia, respectively (Arany et al. 2008).

There is ample evidence that several different mechanisms are responsible for the endurance exercise-dependent activation of PGC-1α, including activation of its expression and/or post-translational modifications (*e.g.* phophorylation, lysine deacetylation, and arginine methylation) (Fig. 1.3). AMP-activated protein kinase (AMPK) is capable of sensing and transducing changes in cellular energy status, and activates the expression of PGC-1α in an unknown manner (Jaeger et al. 2007, Lee et al. 2006, Terada et al. 2002). However, AMPK is not essential for adaptations of skeletal muscle to endurance exercise since it has been reported that a muscle-specific expression of a dominant-negative form of AMPKα2 fails to block the induction of PGC-1α expression and mitochondrial enzyme activity (Rockl et al. 2008), and genetic deletion of functional AMPK isoforms fails to block exercise-induced PGC-1α gene expression in skeletal muscle (Jorgensen et al. 2005). Mechanical stress phosphorylates p38 mitogen-activated protein kinase (p38 MAPK) and is responsible for an increased calcium influx into the muscle cell, leading to phosphorylation and activation of calcineurin and calmodulin dependent kinases. There is numerous evidence supporting the view that p38 MAPK (Akimoto et al. 2005, Knutti et al. 2001, Puigserver et al. 2001) and calcium signalling (Handschin et al. 2003) play an important role in phosphorylating and activating PGC-1α. Moreover, SIRT1 activity provoked by exercise appears to activate PGC-1α through deacetylation (Gurd et al. 2009, Rodgers et al. 2005) and finally, it has been suggested that reactive oxygen species (ROS) also play a role in phosphorylating PGC-1α (Kang et al. 2009, St-Pierre et al. 2006). In conclusion, it is obvious that the exercise-induced activation of PGC-1α as a master-regulator of mitochondrial

biogenesis and angiogenesis in skeletal muscle needs a precise coordination of all the aforementioned regulatory pathways.

Fibre phenotype conversion. A motor unit is defined as the functional unit of a skeletal muscle, consisting of one motoneuron which innervates a collection of fibres of relatively similar mechanical, biochemical, and metabolic properties (Gordon and Pattullo 1993). The motor unit firing frequency determines both the amplitude and duration of Ca^{2+} transients in skeletal muscles. This specific transient change in cytosolic Ca^{2+} regulates selectively slow- and fast-type gene programmes (Chin et al. 1998). Ca^{2+} phosphorylates the Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase, calcineurin (Cn), which in turn dephosphorylates the nuclear factor of activated T-cells (NFAT), enabling its nuclear translocation and DNA binding (Fig. 1.3). Thereby, NFAT induces the activity of slow-fibre-specific gene promoters (*e.g*. myoglobin and troponin I slow). Furthermore, chemically inhibition of Cn in rats resulted in slow-to-fast fibre transformation (Chin et al. 1998), and finally mice lacking the *Cn* gene showed a decreased MYH-1 composition compared to wild-type mice (Parsons et al. 2004). In contrast, other studies revealed that Cn is not solely responsible for the skeletal muscle phenotype changes in response to exercise (Murgia et al. 2000, Swoap et al. 2000) indicating that the signalling pathways affecting the skeletal muscle phenotype is redundant.

Exercise-induced angiogenesis. In 1977, Anderson and Henriksson have observed that endurance exercise training of 40 min cycling on 4 days a week at a power corresponding to ~80% peak oxygen consumption for 8 weeks resulted in a 20% increase in capillary density (Anderson and Henriksson 1977). This was one of the first reports to show that capillary density (*i.e*. angiogenesis) increases after endurance exercise training. Angiogenesis is defined as the formation of new capillaries from existing capillaries, in contrast to vasculogenesis, which is defined as *de novo* formation of the vasculature from precursor cells during development (Prior et al. 2004). It is believed that the expansion of the capillary network after endurance exercise training is based on primarily two mechanisms: 1) capillary intussusception and 2) sprouting angiogenesis (Fig. 1.4).

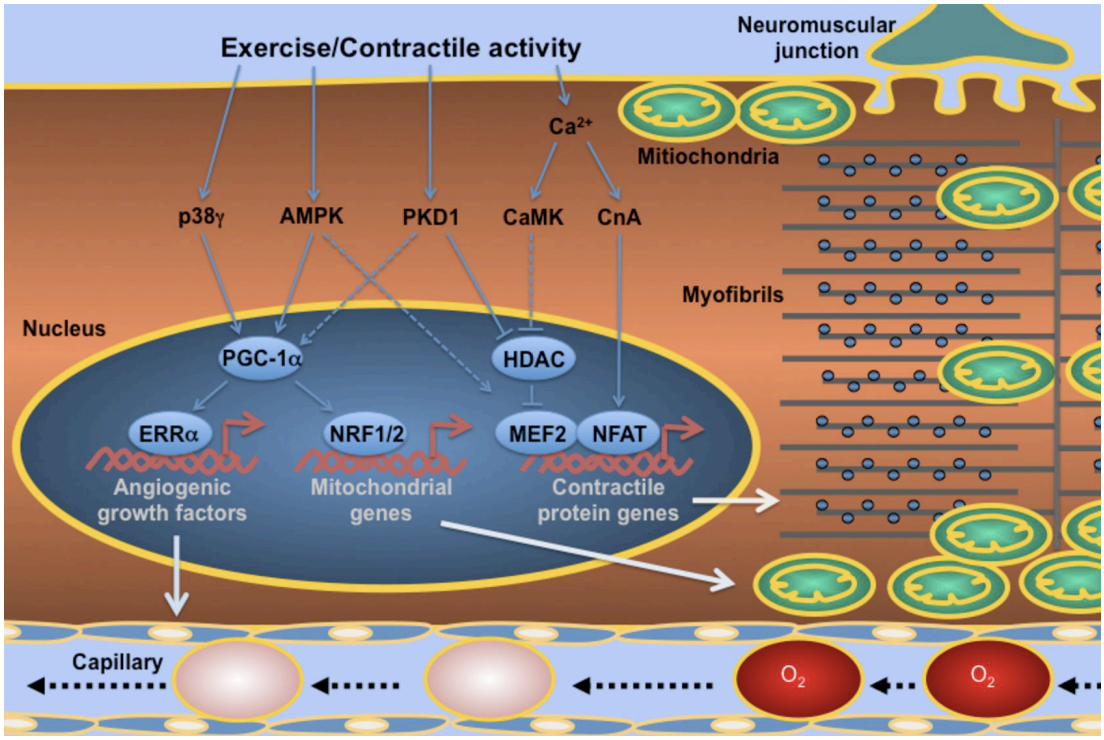

Figure 1.3: Illustration of the signalling and molecular pathways leading to endurance exercise-induced adaptations in skeletal muscle (Yan et al. 2010). The figure depicts an adult skeletal muscle fibre with neuromuscular junction, intracellular metabolic (subsarcolemmal and intermyofibrillar mitochondria) and contractile (myofibrils) apparatuses, and capillary. Solid lines with arrows indicate the findings that have been confirmed by targeted gene deletion animal studies, while dashed lines with arrows indicate findings by transgenic animal models, but not yet by targeted gene deletion studies. AMPK, AMP-activated protein kinase; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CnA, calcineurin A; ERRα, nuclear receptor estrogen-related receptor α; HDAC, histone deacetylase; MEF2, myocyte enhancer factor 2; NFAT, nuclear factor of activated T-cells; NRF1/2, nuclear respiratory factors 1/2; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; PKD1, protein kinase D1.

Intussusception is the process by which a single capillary longitudinally splits into two capillaries within the lumen and sprouting angiogenesis refers to the branching out of endothelial cells from an existing capillary (Egginton 2010, Prior et al. 2004). The capillary network is responsible for the diffusive exchange of oxygen, carbon dioxide, and nutrients between the vascular space and the intracellular space of the muscle fibres. A functional adaptation to endurance exercise training is the increase of oxygen and nutrient supply to the working muscle by expanding the capillary network. The combination of four stimuli is believed to mediate the endurance exercise-induced angiogenesis: growth factors, hypoxia, shear stress, and mechanical stretch (Prior et al. 2004). VEGF has been reported to be the most potent mitogen of endothelial cells that promotes angiogenesis in response to endurance exercise training. Mice with a skeletal muscle-specific *Vegfa* gene deletion displayed a reduced capillary-to-fibre ratio and endurance exercise capacity (Olfert et al. 2009) and an attenuated exercise-induced angiogenesis and improvement of endurance exercise capacity (Olfert et al. 2010). These recent findings indicate the pivotal role of VEGF in the process of exercise-induced angiogenesis. However, an open question is, what the molecular bases underlying the induction of VEGF are. In cell culture, it has been shown that hypoxia increases the VEGF mRNA abundance through hypoxia-inducible factor-1 (HIF-1) activated VEGF promoter activity (Forsythe et al. 1996). HIF-1 is a heterodimeric transcription factor composed of the HIF-1α and HIF-1β subunits and its activity is post-translationally regulated by HIF-1α stabilisation in hypoxic conditions leading to an increased transcriptional activation of several HIF effector genes (Wenger 2000). An acute endurance-exercise session has been shown to increase HIF-1α protein and VEGF mRNA abundances in human skeletal muscle tissue (Ameln et al. 2005). However, in a recent animal study was reported that PGC-1α regulates VEGF and angiogenesis in a HIF-independent manner (Arany et al. 2008). The notion that PGC-1α plays a pivotal role in the regulation of angiogenesis was further supported by the findings that mice with a muscle-specific *PGC-1α* gene deletion showed a blunted endurance exercise-induced VEGF expression and angiogenesis (Chinsomboon et al. 2009, Geng et al. 2010) and mice with a whole-body knockout of the *PGC-1α* gene displayed a reduced VEGF protein expression and a diminished response to acute and chronic endurance exercise training (Leick et al. 2009).

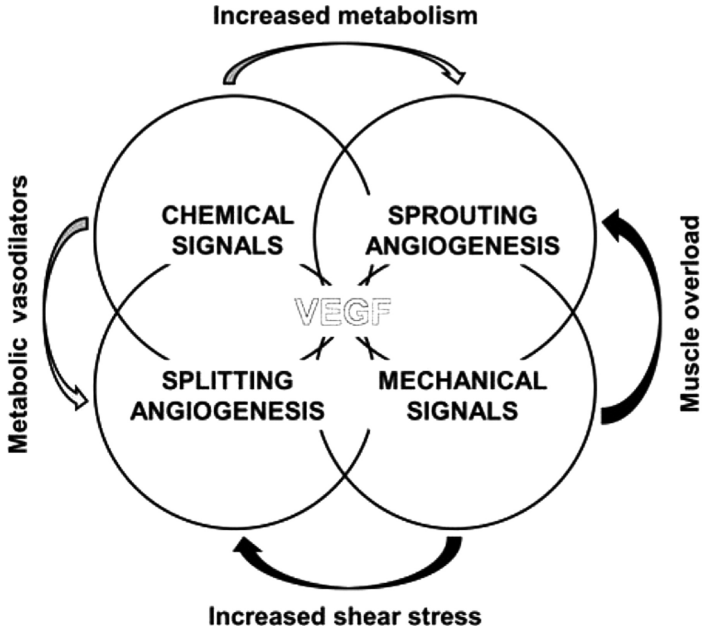

Figure 1.4: Schematic overview of the mechanisms of exercise-induced angiogenesis (Egginton 2010). Skeletal muscle activity induces chemical and/or mechanical signals which lead to increases in the capillary network through vascular endothelial growth factor (VEGF) by two processes, splitting angiogenesis and sprouting angiogenesis.

Adaptations of skeletal muscle in response to resistance exercise

Contrary to endurance exercise, repeated bouts of resistance exercise increase the skeletal muscle fibre cross-sectional area and/or myofibrillar length, skeletal muscle mass, and neuromuscular strength (Tesch et al. 2004). In parallel, overall capillary-to-fibre ratio and mitochondrial volume remain unaffected or even decrease in proportion to muscle hypertrophy (Tesch 1988). These structural adaptations can lead to improved unspecific and/or task-specific strength. Basically, hypertrophy occurs when the rate of protein synthesis is greater than the rate of protein breakdown (Chesley et al. 1992, Phillips et al. 1997). The hypertrophy response to overload relies on a complex myriad of signalling proteins, in which the molecular checkpoint Akt is an important effector of anabolic signals and a dominant inhibitor

of catabolic signals (Nader 2005). The activation of Akt is mediated by the insulin-like growth factor 1 (IGF-1)/phosphatidylinositol-3 kinase (PI3K) pathway, which in turn is stimulated by IGF-1 upon increased muscle loading (Glass 2005, Rennie et al. 2004, Toigo and Boutellier 2006). There are two main pathways downstream of PI3K and Akt to mediate hypertrophy: the Akt/mammalian target of rapamycin (mTOR) pathway, and the Akt/glycogen synthase kinase 3β (GSK3β) pathway (Fig. 1.5). mTOR, a Ser/Thr protein kinase, phosphorylates the ribosomal protein S6 kinase (S6K1/p70^{S6k}) and inhibits the activity of translational repressor eukaryotic initiation factor 4E-binding protein 1 (4E-BP1/PHAS-1), which is a negative regulator of the protein initiation factor eIF4E. Inactivation of GSK3β increases the rate of translation by increasing eIF2B activity in an Akt/mTOR independent way. However, recent studies with pharmacological, genetic, and transgenic approaches reveal that the mTOR signalling pathway can be induced in a PI3-kinase independent way. Based on these findings, the importance of the IGF/PI-3 kinase/Akt signalling pathway in load induced muscle growth has been questioned (Philp et al. 2010). To summarise, evidence exists that the Akt/mTOR as well as the Akt/GSK3β pathways have major effects on skeletal muscle hypertrophy, but that there are other growth-signalling pathways in skeletal muscle (*e.g.* mitogen-activated protein kinase [MAPK] and calcineurin signalling pathways), which possibly interact with each other leading to a significant redundancy.

Adaptations of skeletal muscle in response to concurrent training

It has been postulated that training to improve strength is negatively affected by concurrent endurance exercise. Specifically, it has been shown that simultaneous strength and endurance exercise impairs strength gains compared with resistance exercise alone (Hickson 1980). At the molecular level, this phenomenon has been explained by a distinct transcriptional and (post-) translational mechanism of adaptation induced by resistance and endurance exercise, with each mode of exercise activating and/or repressing specific subsets of genes and cellular signalling pathways which can interfere with each other (Atherton et al. 2005, Baar 2006, Coffey and Hawley 2007, Hawley 2009, Nader 2006). One molecular mechanism potentially explaining the interference effect proposes that AMPK, activated by endurance exercise, phosphorylates and activates the tuberous sclerosis complex (TSC2, tuberin) (Atherton et al. 2005, Inoki et al. 2003). Activation of TSC2 by

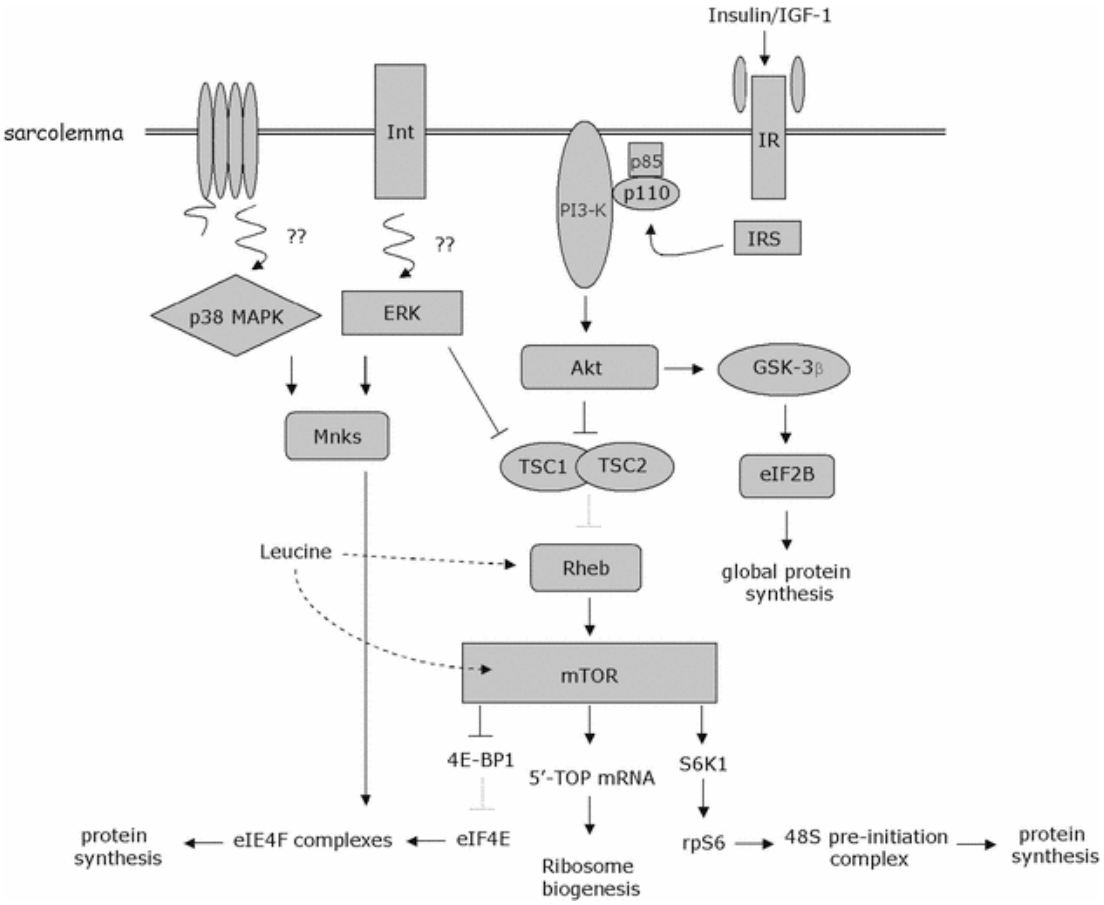

Figure 1.5: Overview of the signalling mechanisms mediating the anabolic response to resistance exercise (Phillips 2009). A multitude of kinases phosphorylates and thus activates or inactivates other kinases which finally lead to increased protein synthesis. 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; eIF, eukaryotic initiation factor; GSK3β, glycogen synthase kinase 3β, Int, integrin, IGF-1, insulin-like growth factor 1; IR, insulin receptor; IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin; PI3-K, phosphatidylinositol-3 kinase; TSC, tuberous sclerosis complex; Rheb, rat homologue enriched in brain; S6K1, ribosomal protein S6 kinase.

AMPK is dominant over the resistance exercise-induced Akt-mediated inactivation, leading to the inactivation of mTOR and therefore to a decreased rate of protein synthesis. However, although it seems that endurance and strength exercise performed at the same time do not promote optimal activation of pathways that simultaneously promote both anabolic and endurance responses, the underlying molecular mechanisms are only poorly understood.

Effects of inactivity on skeletal muscle

A 20-day period of bed rest by young, healthy male is associated with muscle wasting, bone loss, insulin resistance, reduced peak cardiac output and stroke volume, orthostatic intolerance, decreased immune function, and decreased physical capacity (Saltin et al. 1968). Moreover, it is not only extreme situations such as bed rest, which result in such severe impairments in physiological function. A more recent study reported that reducing daily activity level from 10'000 to 1'300 steps per day for 2 weeks, in the absence of a structured exercise programme, results in decreases in insulin-stimulated skeletal muscle Akt phosphorylation, peripheral insulin sensitivity, leg lean mass, and whole-body peak oxygen consumption (Krogh-Madsen et al. 2010). Furthermore, inactivity has been associated with impaired lipid handling (McGarry 2002), skeletal muscle capillary regression (Prior et al. 2004), mitochondrial dysfunction (Timmons et al. 2006), and a coordinated down-regulation of PGC-1α and genes involved with mitochondrial metabolism (including muscle substrate delivery genes) (Timmons et al. 2006). However, it is important to note that molecular changes that initiate adaptations to detraining are not the opposite molecular adaptations to exercise training. For instance, mitochondrial biogenesis requires about 1'500 different nuclear-encoded proteins, whereas the loss of mitochondria requires a decline in mitochondrial membrane potential and mitochondrial autophagy by lysosomes, implying different molecular mechanisms. Moreover, skeletal muscle hypertrophy reveals a very complex sequence of events that are not the simple reverse of the muscle loss process.

Skeletal muscle function and chronic diseases

A multitude of molecular, structural, and functional impairments leading to limited oxidative exercise capacity have been attributed to several chronic diseases. Chronic heart failure, chronic obstructive pulmonary disease, and type 2 diabetes are associated with skeletal muscle dysfunction as indicated by reduced skeletal muscle fibre capillarisation, oxidative enzyme activity, mitochondrial volume, and/or mitochondrial phosphorylation potential (Drexler et al. 1992, Duscha et al. 1999, He et al. 2001, Jakobsson et al. 1995, Jobin et al. 1998, Kelley et al. 2002, Larsson et al. 1999, Sala et al. 1999). Consistent with the decreased skeletal muscle oxidative capacity, peak cardiac output, whole-body peak oxygen consumption, and endurance capacity are impaired (Esposito et al. 2010, Regensteiner et al. 2009, Ribisl et al. 2007, Serres et al. 1998, Wilson et al. 1984). Mitochondrial dysfunction is not only a common feature of many chronic diseases but is also associated with muscle atrophy (Romanello and Sandri 2010) and ageing (Dufour and Larsson 2004, Figueiredo et al. 2008). At the molecular level, reports indicate that patients with diabetes exhibit coordinated reduced expression of multiple genes involved in oxidative metabolism (Mootha et al. 2003, Patti et al. 2003). Based on the observed skeletal muscle dysfunctions in chronic diseases, some scientists have hypothesised that these molecular, structural, and functional impairments account for the aetiology of several chronic diseases. For instance, it has been suggested that skeletal muscle mitochondrial deficiency and/or dysfunction causes insulin resistance, which leads to diabetes (Lowell and Shulman 2005, Schrauwen-Hinderling et la. 2007). However, the precise mechanism behind this theory remains unclear. It has been hypothesised that mitochondrial deficiency/dysfunction impairs the ability of muscle to oxidise fatty acids, and leads to intramuscular lipid accumulation and insulin resistance (Kelley et al. 2002, Lowell and Shulman 2005, Morino et al. 2006). In contrast, Holloszy (2009) recently estimated that even if mitochondrial capacity was reduced, skeletal muscle still would contain sufficient mitochondria to allow a ~150-fold increase in oxygen uptake per kg of muscle under exercise conditions. In fact, Boushel et al. (2007) reported that although there was a decrease in mitochondrial content, the remaining mitochondria functioned normally. Based on theses findings the relationship between mitochondrial deficiency and insulin resistance has been questioned (Holloszy 2009). Thus, the important question for

further studies is whether individuals with type 2 diabetes have a genetic predisposition to low muscle oxidative capacity, or whether the impaired mitochondrial capacity arises from environmental/lifestyle-related factors. Recently, it has been suggested that the latter is the more likely scenario (Hawley and Lessard 2007, Holloszy 2009).

Type 1 diabetes. The American Diabetes Association defines type 1 diabetes as a metabolic disease characterised by hyperglycaemia resulting from a cellular-mediated autoimmune destruction of the β-cells of the pancreas (American Diabetes Association - Position statement 2006). Although type 1 diabetes commonly occurs in childhood and adolescence, it can appear at any age, even in the 8th and 9th decades of life. The rate of β-cell destruction varies during the individual aetio-pathology finally leading to little or no insulin secretion. Thus, individuals with type 1 diabetes are dependent on insulin therapy and are at high risk to develop keto-acidosis, which is a potentially deadly metabolic complication. Multiple genetic predispositions and poorly defined environmental factors are believed to cause the autoimmune destruction of β-cells. However, there is growing evidence that insulin resistance is not only present in type 2 diabetes but is also a hallmark of type 1 diabetes and that also decreased β-cell function manifests in both forms of diabetes. These findings led to the assumption that the aetiology and conditions of type 1 and 2 diabetes are blurring and overlapping (Wilkin 2001, Donath 2004). In line with this notion it has been suggested that the gain in visceral fat mass occurring in both types of diabetes might contribute to the insulin resistant state (Wilkin 2001). In fact, there are several reports that individuals with type 1 diabetes display decreased insulin sensitivity compared to healthy controls (Nadeau et al. 2010, DeFronzo et al. 1982a,b, Greenbaum 2002, Dabelea et al. 2003). Although it is not clear up to now, if a decreased insulin sensitivity is associated with reduced mitochondrial capacity, one study reported a reduced mitochondrial capacity in conjunction with increased glycolytic flux in men with type 1 diabetes representing a metabolic shift common to chronic metabolic diseases (Crowther et al. 2003). However, results at the systemic level are contradicting since some authors reported decreased peak oxygen uptake and/or reduced submaximal cardiac output (Nadeau et al. 2010, Gusso et al. 2008, Poortmans et al. 1986), yet, other investigators showed that peak oxygen uptake is not affected in patients with type 1 diabetes (Fritzsche et al. 2008, Harmer et al. 2008, Veves et al. 1997). Taken together, there exist only limited evidence to show whether aerobic function is impaired in type 1 diabetes.

Linking physical inactivity with chronic diseases

Although it is challenging to prove causality between physical inactivity and chronic diseases, it is important to note that in both conditions impairments of the skeletal muscle are based on the same molecular, cellular, structural, and functional changes. In addition, there is not doubt that regular exercise is a fundamental strategy in combating many life-style disorders. Exercise induces adaptations in almost every organ system, such as circulatory, endocrine, neural, skeletal muscle, connective tissue, gastrointestinal, immune, and kidney leading to health improvements (Fig. 1.1, Booth and Laye 2009). Although exercise affects all organs in the body, major positive impacts are believed to result directly from skeletal muscle adaptations (Kraus et al. 2001, Yan et al. 2010). Therefore, in order to gain a better understanding of the mechanisms underlying skeletal muscle plasticity, it is fundamental to identify which mechano-biological condition leads to what molecular/cellular response, and how this molecular/cellular response relates to the structural, contractile, and metabolic adaptation (Toigo and Boutellier 2006). An improved understanding of the molecular and signalling mechanisms underlying exercise-induced skeletal muscle adaptation will provide invaluable information for designing therapeutic, exercise interventions for many of the chronic diseases that affect people worldwide.

Objectives

High aerobic function is related to physical activity whereas low aerobic function is related to physical inactivity and various metabolic diseases. In order to shed light upon the relationship of aerobic function and type 1 diabetes, we used an integrative approach to investigate factors that may limit oxidative capacity and aerobic exercise performance in young untrained women with type 1 diabetes (Chapter 2). For a better understanding of the plasticity of the skeletal muscle aerobic function we aimed at establishing a cell culture model to closely recapitulate the plastic changes in gene expression as observed in aerobically trained skeletal muscles of mice (Chapter 3). Typically, aerobic function can be improved by repeated endurance exercise but not by resistance exercise. To overcome the specificity of this adaptive response we aimed at designing a new resistance exercise model with superimposed stimuli to induce endurance type adaptations and to test its effectiveness in humans (Chapter 4). In addition, we aimed at analysing the molecular bases underlying these adaptations (Chapter 5).

Chapter 2

Oxidative capacity is affected by glycaemic status in young untrained women with type 1 diabetes but is not impaired relative to healthy untrained women

Flurin Item[1,2*], Susanne Heinzer-Schweizer[3*], Michael Wyss[3], Piero Fontana[1], Roger Lehmann[4], Anke Henning[3], Markus Weber[5], Peter Boesiger[3], Urs Boutellier[1,2], and Marco Toigo[1,2] ([*] Authors contributed equally to this work)

[1] Exercise Physiology, Institute of Human Movement, ETH Zurich, Zurich, Switzerland; [2] Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland; [3] Institute for Biomedical Engineering, University and ETH Zurich, Zurich, Switzerland [4] Department of Endocrinology, Diabetes and Clinical Nutrition, University Hospital Zurich, Zurich, Switzerland; [5] Department of Visceral and Transplantation Surgery, University Hospital Zurich, Zurich, Switzerland

Submitted to: American Journal of Physiology - Regulatory, Integrative and Comparative Physiology (In revision)

Introduction

Oxidative capacity depends on the transport of oxygen from ambient air to the mitochondrial respiratory chain, and relies on a coordinated action between several processes (*i.e.* ventilation, blood flow, diffusion), which depends on distinct structures (*i.e.* lung, heart, capillaries, muscle fibres, mitochondria). Maximal oxygen uptake (aerobic exercise capacity) is determined by convective (perfusive) and diffusive oxygen transport capacity (Roca et al. 1992). Whereas convective oxygen transport capacity (*i.e.* oxygen delivery) is given by the product of arterial oxygen content and cardiac output, diffusive oxygen transport capacity is determined by arterial-mixed venous oxygen difference, which in turn relies on capillary supply and mitochondrial capacity (Saltin et al. 1968). Aerobic exercise capacity is reduced in several chronic diseases, *e.g.* chronic heart failure (Esposito et al. 2010), chronic obstructive pulmonary disease (Mador and Bozkanat 2001), and type 2 diabetes (Ribisl et al. 2007).

While the relationship between reduced aerobic exercise capacity and type 2 diabetes is widely accepted, the available data for type 1 diabetes are contradictory. Some authors report decreased peak oxygen uptake and/or reduced submaximal cardiac output (Gusso et al. 2008, Nadeau et al. 2010, Poortmans et al. 1986), yet, other investigators showed that peak oxygen uptake is not affected in patients with type 1 diabetes (Fritzsche et al. 2008, Harmer et al. 2008, Veves et al. 1997). However, none of these authors have concurrently investigated both perfusive and diffusive components determining aerobic exercise capacity. Using ^{31}P-Magnetic Resonance Spectroscopy (MRS), Crowther *et al.* (2003) found that mitochondrial capacity (*i.e.* the maximal rate of oxidative ATP synthesis) is reduced in male patients with type 1 diabetes compared to healthy controls, and they suggested that reduced muscle oxidative capacity in conjunction with increased glycolytic flux represents a metabolic shift common to chronic metabolic diseases (obesity, type 1 and 2 diabetes). However, Holloszy (2009) recently estimated that even if mitochondrial capacity was reduced, skeletal muscle still would contain sufficient

mitochondria to allow a ~150-fold increase in oxygen uptake per kg of muscle under exercise conditions. Therefore, it is questionable whether and to which extent reduced mitochondrial capacity may limit aerobic exercise capacity. Would reduced mitochondrial capacity indeed lead to reduced aerobic exercise capacity, the decrease in aerobic exercise capacity should manifest itself in a decreased diffusive oxygen transport capacity.

Taken together, it is currently not well understood a) whether oxidative capacity is reduced in patients with type 1 diabetes at all, b) which components (*i.e.* perfusive and/or diffusive) contributing to aerobic exercise capacity are reduced, c) whether the proposed reduced mitochondrial capacity (Crowther et al. 2003) translates into reduced diffusive oxygen transport capacity, and d) if glycaemic status influences mitochondrial capacity. In this study we concurrently investigated several components of convective and diffusive oxygen transport capacity including muscle mitochondrial capacity in young women with type 1 diabetes and healthy women of similar age and physical activity level. To test if aerobic exercise capacity and its determinants are reduced in patients with type 1 diabetes, we then compared maximal oxygen uptake, maximal cardiac output, calculated arterio-venous oxygen difference, ^{31}P-MRS-derived maximal rates of calf muscle oxidative ATP synthesis, vastus lateralis and soleus muscle fibre metabolic phenotype and capillarisation between the two groups. In addition, we assessed ventilatory threshold and endurance capacity to investigate differences in submaximal exercise capacity between the two groups.

Design and Methods

Participants

We recruited 29 young sedentary asymptomatic women (CON) and 9 women of similar age and activity level with type 1 diabetes (DIA). Mean age was 24.1 (SD 4.2) years for CON and 26.9 (5.2) years for DIA without significant difference. The participants' anthropometric data are presented in Table 2.1. Women with type 1

diabetes had no diabetic complications or coexisting cardiovascular diseases, and were classified as C-peptide negative (< 0.5 $\mu U \cdot ml^{-1}$). The duration of diabetes was 13.1 (6.6) years. Relative (per kg body mass) mean total daily insulin dose was 0.6 (0.3) $units \cdot kg^{-1}$. Glycosylated haemoglobin A1c (HbA1c) was 5.3 (0.2) (range 5.0-5.7%) and 7.6 (0.4) % (range 6.9-8.2%) for CON and DIA, respectively, and different between groups ($P < 0.001$). Participants in the CON group showed no sign of either impaired glucose tolerance [fasting glucose concentration: 4.49 {0.26} $mmol \cdot l^{-1}$, blood glucose concentration 2 h post glucose load:4.92 {1.19} $mmol \cdot l^{-1}$ (Unwin et al. 2002)] or calculated insulin resistance [homeostasis model assessment {HOMA-IR (Matthews et al. 1985)}: 1.4 {0.4}]. All participants were untrained (less than 1 h of physical activity per week). After completing a routine health questionnaire the participants were informed about the applied procedures and the associated risks. Informed written consent was obtained from all participants. The experimental protocol was approved by the ethics committee of the canton of Zurich, and the study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki for human experimentation.

Magnetic resonance spectroscopy and imaging measurements

^{31}P-MRS scans were acquired at rest and during isometric muscle contraction using a 3 T whole body Philips Achieva Scanner (Philips Healthcare, Best, The Netherlands) with a transmit/receive surface coil (diameter: 0.14 m) tuned to 51.8 MHz. The MR-compatible ergometer setup was compound of a dynamometer with an integrated strain gauge (Sensory-Motor Lab, ETH Zurich, Switzerland) for force measurements and a real-time visual feedback system for providing the participants with information on the level of exerted force relative to maximal voluntary force. ^{31}P-MRS spectra of the triceps surae muscles were acquired using a pulse-acquire technique and an adiabatic hyperbolic secant excitation pulse. The test protocol consisted of isometric plantarflexion at 85% of the maximal force acting on the pedal (MVF_P) for 30 s. Sequence parameters included: repetition time = 1.5 s, 3 signal averages per time point, 2048 sample points, 5 dummy scans; 20 spectra were recorded before the exercise for saturation correction; total number of spectra: 84. Recovery spectra were measured after cessation of the isometric contraction for 324 s. The reasons for this test protocol were that 1) pH remains close to 7.0, which is mandatory to derive valid phosphocreatine (PCr) recovery rates (Arnold et al. 1984),

2) at 85% MVF_P motor unit recruitment of large muscles is complete (DeLuca et al. 1996), and 3) the obtained mean decrease in PCr concentration is sufficiently high to calculate PCr recovery rate (k_{PCr}) (Meyer 1988).

Processing: Free-induction decays were zero-filled, line-broadened, Fourier-transformed into spectra, and fitted using *tdfdfit* (Slotboom et al. 1998). Inorganic phosphate (P_i) and PCr concentrations were calculated using ATP as internal reference standard, assuming a concentration of 8.2 mM (Arnold et al. 1984). The saturation factor in the dynamic spectra was determined by the ratio of the relaxed spectra to the partially saturated spectra acquired prior to the plantarflexion exercise. We calculated k_{PCr}, which is an indirect marker for the maximal rate of oxidative ATP synthesis (Sahlin 1978), using a mono-exponential fitting procedure. Oxidative capacity was calculated as the product between k_{PCr} and resting PCr concentration (Conley et al. 2000). Intracellular pH-values were calculated from the chemical shift of P_i according to the modified Henderson-Hasselbach equation (Petroff et al. 1988).

Skeletal muscle biopsy analyses

Sample preparation. We obtained percutaneous biopsies from the middle region of the nondominant *soleus* and *vastus lateralis* muscles, using a ProMag Ultra device and 14 gauge needles (Angiotech Pharmaceuticals, Gainesville, FL, USA). Muscle tissue was mounted in an embedding medium (Tissue-Tek®, Sakura, Zoeterwoude, The Netherlands), snap frozen in isopentane cooled to –160 °C with liquid nitrogen. We stained the serial cryocut cross-sections using the myofibrillar *adenosine-triphosphatase* (mATPase) method according to Guth and Samaha (1970) with minor modifications (Muentener 1979). Muscle fibres were classified according to their myosin heavy chain (MYH) isoform into MYH-1 and MYH-2. For the analysis of oxidative enzyme activity, we incubated consecutive sections in media containing *cytochrome c oxidase*. The monoclonal mouse anti-human CD31 endothelial cell antibody (DAKO, Carpinteria, Canada, 1:600 dilution) was used as a marker for muscle, and capillary-to-fibre ratio was calculated by dividing the number of CD31-positive cells by the number of muscle fibres. For all histochemical and immunohistochemical analyses, we used the NIH Image J software (1.410, National Institutes of Health, Bethesda, MD, USA). *Cytochrome c* activity was determined

from the measured mean optical density pixel values of the muscle fibres normalised to the background pixel values on the same section, and reported in arbitrary numbers. For all fibre analyses, only fibres fully encircled by adjacent fibres were evaluated, and measurements were made for at least 50 of each of the main fibre types (*i.e.* MYH-1 and MYH-2). Previous studies investigating the skeletal muscle fibre sample size required for a reliable, valid representation of an individual's average fibre area and capillary-to-fibre ratio, showed that 50 fibre measurements per individual for type 1 and 2 fibres and capillary contacts are sufficient to characterise type 1 and 2 fibre areas and capillary-to-fibre ratio of an individual (McCall et al. 1998, Porter et al. 2002).

Cardiac output and oxygen consumption measurements

We used Innocor™ (Innovision, Odense, Denmark) to estimate cardiac output by inert gas rebreathing and oxygen consumption by breath-by-breath ergospirometry during a graded cycling exercise test (GXT), as previously described (Fontana et al. 2009). Participants started cycling at 50 W and power was increased by 25 W every 2 min until volitional exhaustion. Arterio-venous oxygen difference was calculated by dividing oxygen consumption by cardiac output. Ventilatory threshold was determined as the power corresponding with a disproportionate increase in minute ventilation during the GXT.

Endurance capacity

On a separate day, we used a constant-load cycling exercise test (CLT) to assess time to exhaustion at submaximal power as an indicator of endurance capacity. Power of the initial warm-up stage was 40% maximal power of the first GXT. After 1 min, we increased power to 60% peak power (for 2 min) and then to 85% maximal power. Power at 85% maximal power was sustained until volitional exhaustion, *i.e.* the point in time at which the participants stopped pedalling or were no longer able to maintain pedal rate within the required limits.

Dual-energy X-ray absorptiometry

We performed dual-energy X-ray absorptiometry (DXA) measurements using Lunar iDXA™ (GE Healthcare, Madison, WI, USA).

Blood analyses

Plasma glucose concentrations were determined by an automated hexokinase method (HK Unit-Kit III, Roche, Basel, Switzerland), and serum insulin concentrations were determined by radioimmunoassay (Insulin ct-kit, Cisbio Bioassays, Bagnols-sur-Cèze, France). HbA1c was immunochemically determined with a DCA 2000® device (Bayer, Leverkusen, Germany). Furthermore, CON participants performed a standard 75 g-oral glucose tolerance test (OGTT) in the morning after an overnight (12 h) fast.

Statistical analyses

Data are presented as mean and standard deviation (SD). Group differences between CON and DIA were tested for statistical significance by *t*-tests for independent samples, and equality of variances was checked using Levene's test. The level of significance was set to $P < 0.05$. For all statistical analyses, SPSS 16.0 statistical software (SPSS, Chicago, IL, USA) was used.

Results

Calf muscle mitochondrial capacity

As indicated by the identical post-exercise k_{PCr} for CON and DIA [0.0307 (0.0070) and 0.0309 (0.0058) s^{-1}, respectively], calculated from the PCr recovery over time (Fig. 2.1A), calf muscle mitochondrial capacity was not different (P = 0.930) between the two groups. pH did not affect PCr recovery, as shown by the independency of k_{PCr} from pH (Fig. 2.1B). For both, CON and DIA, the end-exercise pH values were close to 7.0 and not different from the values at rest [CON: 7.03 (0.06) vs. 7.03 (0.02), P = 0.846; DIA: 7.02 (0.10) vs. 7.03 (0.01), P = 0.707]. Between groups, there were no differences in either resting (P = 0.262) or end-exercise pH (P = 0.845, Fig. 2.1C). The calculated calf muscle oxidative capacity [1.15 (0.28) vs. 1.19 (0.30) $mM \cdot s^{-1}$, P = 0.702], resting Pi concentrations [4.87 (0.75) vs. 4.67 (0.57) mM, P = 0.467] and resting PCr concentrations (P = 0.669, Fig. 2.1A) were similar between CON and DIA.

Skeletal muscle fibre properties

For both, *vastus lateralis* and *soleus* muscles, no differences in capillary-to-fibre ratio and MYH-1 fibre percentage were found between CON and DIA (Fig. 2.2A, B). The oxidative fibre phenotype was not different between CON and DIA, as indicated by the similar *cytochrome c oxidase* activity in MYH-1 and MYH-2 fibres (Fig. 2.2C). In general, muscle fibre diameters tended to be smaller for CON relative to DIA (Fig. 2.2D). However, only the *vastus lateralis* muscle MYH-2 mean fibre diameter was significantly (P = 0.006) smaller in CON than in DIA (Fig. 2.2D).

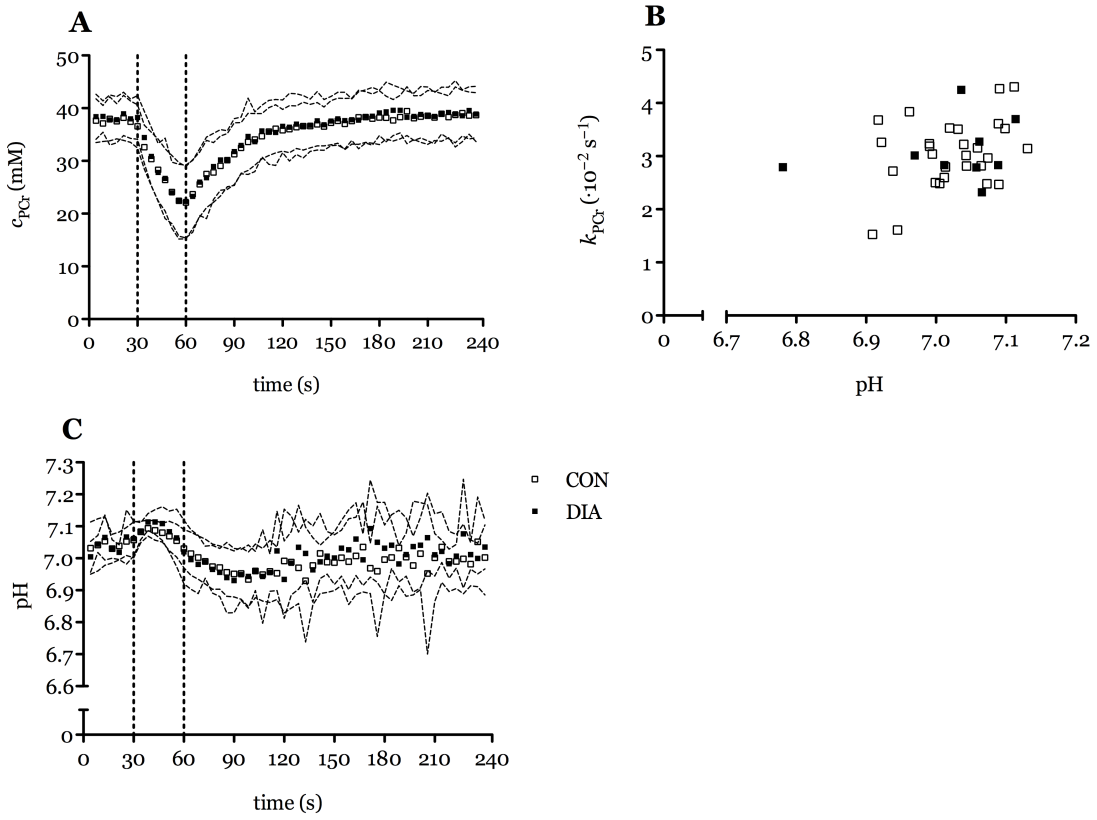

Figure 2.1: Phosphocreatine concentration (c_{PCr}) during isometric plantarflexion exercise (A), relationship between PCr recovery rate constant (k_{PCr}) and end-exercise pH (B), and time course of pH during isometric plantarflexion exercise (C). Squares and dashes represent means and SD, respectively, for 28 and 9 women in CON and DIA, respectively. One individual in the CON group was excluded from the k_{PCr} data set because of a k_{PCr} value more than 4 SD from the mean. Dashed vertical lines in (A) and (C) indicate the start and end of exercise. CON, healthy women; DIA, women with type 1 diabetes. No significant differences were found.

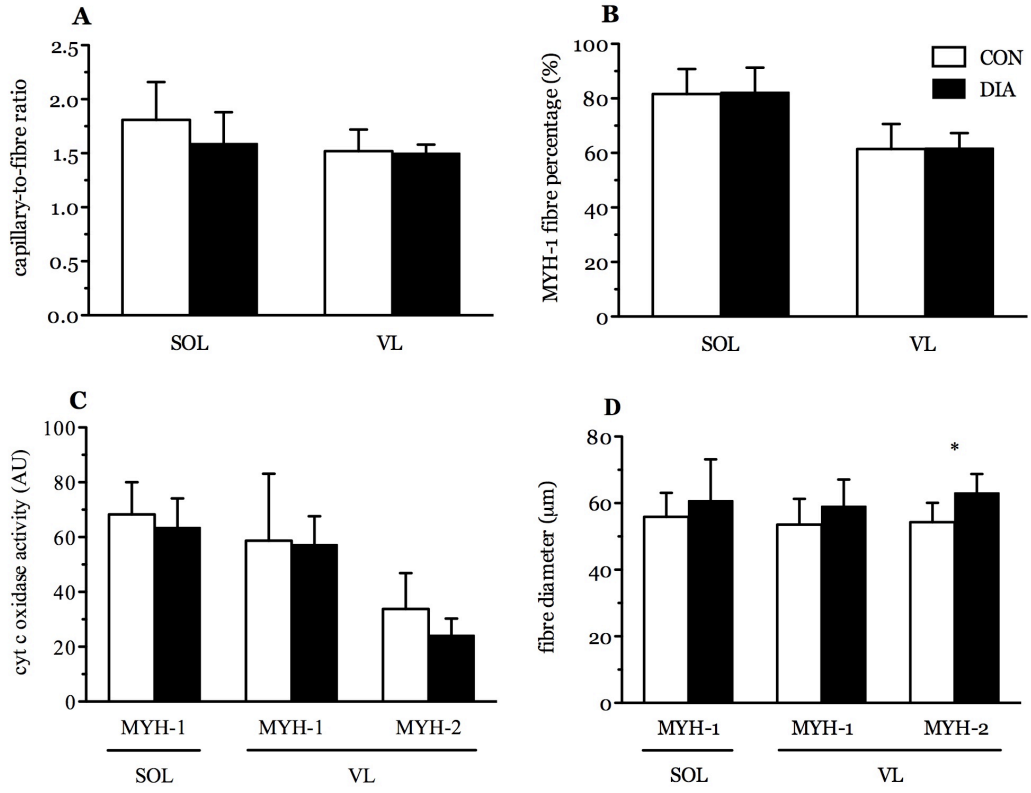

Figure 2.2: Capillary-to-fibre ratio (A), MYH-1 fibre percentage (B), *cytochrome c* (cyt c) *oxidase* activity (C), and fibre diameter (D) of *soleus* (SOL) and *vastus lateralis* (VL) muscle fibres. Bars and error bars represent mean values and standard deviations, respectively. Only datasets meeting the described quality criteria were included (*soleus* muscle MYH-1 fibre percentage: 9 CON and 5 DIA; *vastus lateralis* muscle MYH-1 fibre percentage: 13 CON and 6 DIA; *cyt c oxidase* activity and fibre diameter: 14 CON and 6 DIA; *soleus* muscle capillary-to-fibre ratio: 15 CON and 5 DIA; *vastus lateralis* capillary-to-fibre ratio: 14 CON and 6 DIA). AU, arbitrary units; MYH-1, myosin heavy chain isoform type 1; MYH-2, myosin heavy chain isoform type 2; CON, healthy women; DIA, women with type 1 diabetes. *$P < 0.05$.

Oxygen consumption, cardiac output, and arterio-venous oxygen difference

Oxygen consumption, cardiac output, and calculated arterio-venous oxygen difference were not different between CON and DIA at maximal and submaximal power during a GXT (Fig. 2.3).

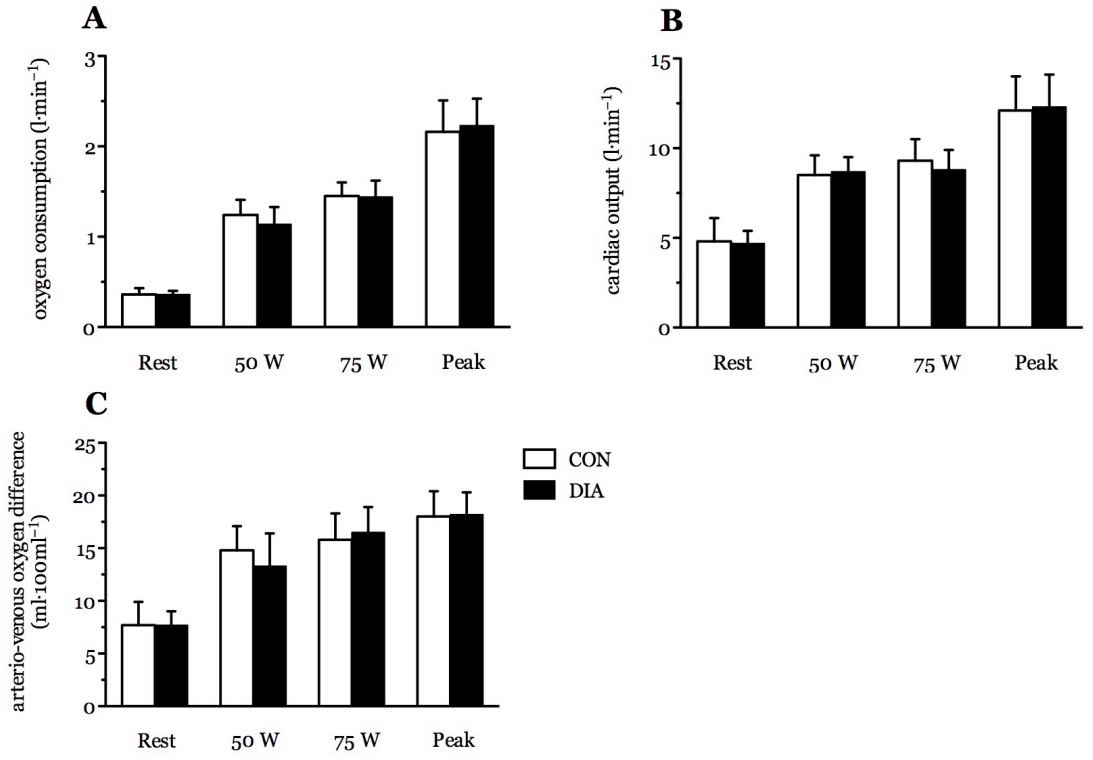

Figure 2.3: Oxygen consumption (A), cardiac output (B), and calculated arterio-venous oxygen difference (C) at submaximal and maximal power during a GXT to exhaustion. Bars and error bars represent mean values and SD, respectively, for 29 healthy women (CON) and seven women with type 1 diabetes (DIA). Two individuals in the DIA group did not agreed to participate in a GXT. GXT, graded cycling exercise test. No significant differences were found.

Exercise performance, ventilatory threshold and endurance capacity

We found no difference in maximal power output [163 (26) vs. 171 (25) W, P = 0.486] and ventilatory threshold [124 (18) vs. 125 (17) W, P = 0.942] between CON and DIA during a GXT. Furthermore, cycling endurance capacity was not different between the two groups, as indicated by the similar cycling time to exhaustion [CON: 532 (212) s, DIA: 471 (119) s, P = 0.475] during a CLT at submaximal power.

Effect of glycaemic control on mitochondrial capacity and cardiac output

HbA1c was negatively correlated with k_{PCr} (R^2 = 0.475, P = 0.040, Fig. 2.4A) and peak cardiac output (R^2 = 0.742, P = 0.013; Fig. 2.4B) in women with type 1 diabetes.

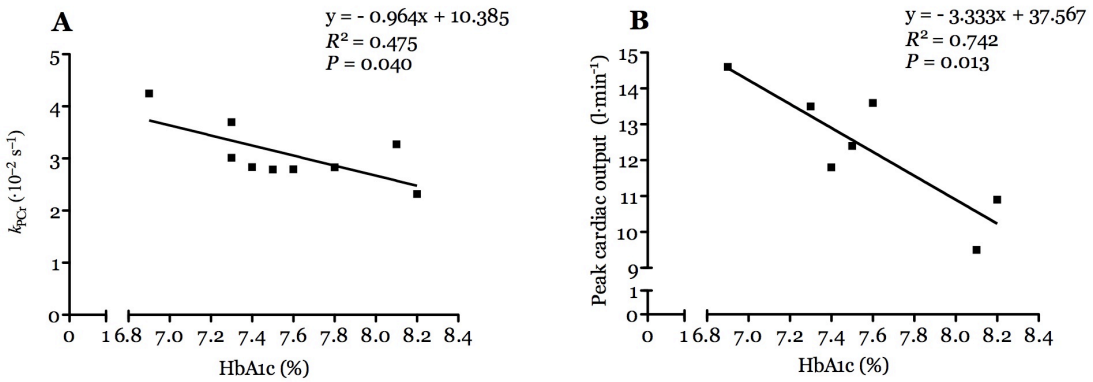

Figure 2.4: PCr recovery rate constant (k_{PCr}) (A) and peak cardiac output (B) as a function of glycosylated haemoglobin A1c (HbA1c) in nine (A) and seven (B) young untrained women with type 1 diabetes.

Body composition

Both, total and segmental lean and fat masses, were lower in CON relative to DIA, while % body fat was the same in both groups (Tab. 2.1).

Table 2.1: Body composition of study participants

	CON	DIA
Body mass (kg)	61.4 (6.5)	69.4 (8.6) **
Body height (m)	1.68 (0.06)	1.68 (0.06)
Body mass index ($kg \cdot m^{-2}$)	21.8 (2.1)	24.6 (3.0) **
Body fat (%)	31.6 (5.4)	35.0 (4.8)
Body fat mass (kg)	19.0 (4.5)	23.9 (5.6) *
Lean mass (kg)	40.6 (4.1)	43.7 (4.1)
Abdominal fat mass (kg)	1.35 (0.54)	1.78 (0.71)
Abdominal lean mass (kg)	2.46 (0.29)	2.70 (0.26) *
Gluteofemoral fat mass (kg)	4.68 (0.98)	5.83 (1.30) **
Gluteofemoral lean mass (kg)	5.53 (0.58)	5.92 (0.78)
Leg fat mass (kg)	7.97 (1.68)	10.32 (2.47) **
Leg lean mass (kg)	14.10 (1.60)	15.44 (1.83) *
Thigh fat mass (kg)	6.21 (1.26)	8.18 (2.03) **
Thigh lean mass (kg)	10.62 (1.23)	11.70 (1.41) *
Lower leg fat mass (kg)	1.67 (0.34)	2.15 (0.53) **
Lower leg lean mass (kg)	3.39 (0.48)	3.74 (0.55)

Data are expressed as means (SD) for 29 healthy women (CON) and 9 women with type 1 diabetes (DIA). *$P < 0.05$, **$P < 0.01$.

Discussion

The aim of the present study was to determine whether oxidative capacity is reduced in women with type 1 diabetes and whether their glycaemic status influences mitochondrial capacity. We found that in women with type 1 diabetes, glycaemic status affected mitochondrial capacity and cardiac output, but that maximal oxygen uptake, maximal cardiac output, calculated arterio-venous oxygen difference, *in vivo* skeletal muscle mitochondrial capacity, muscle fibre metabolic phenotype, and capillarisation were not different between women with type 1 diabetes and healthy women of similar age and physical activity level. Analogous to maximal aerobic capacity, we found that ventilatory threshold and endurance capacity were similar between the two groups. Our results indicate that glycaemic status affects mitochondrial capacity in women with type 1 diabetes, but that neither maximal nor submaximal aerobic capacity is reduced in young women with type 1 diabetes relative to healthy controls.

To obtain maximal rates of oxidative ATP synthesis we applied a test protocol that decreased skeletal muscle PCr concentration but left intracellular pH close to 7.0 (Fig. 1), the latter being crucial to derive valid PCr recovery rates (Arnold et al. 1984). Our finding that the maximal rate of oxidative ATP synthesis (mitochondrial capacity) was similar between women with type 1 diabetes compared to healthy controls (Fig. 2.1) conflicts with that of Crowther et al. (2003), who reported slower PCr recovery rates in men with type 1 diabetes relative to age-matched controls. Three reasons could possibly help to explain the divergent findings. A first reason might be that Crowther et al. (2003) determined oxidative capacity from isometric dorsiflexions while we used isometric plantarflexions. Thus, differences in oxidative metabolism between dorsiflexion and plantarflexion muscles might lead to divergent findings. But given the fact that MYH-1 fibre type proportion of tibialis anterior muscle lies between that of *soleus* and *gastrocnemius* muscles (Saltin et al. 1977), it seems unlikely that differences in fibre type distribution represent a reason for discrepancy. A second possible reason might be that in the study by Crowther et al.

(2003) the participants were not able to sustain the target force of 70-75% MVF_P for 30 s [Figure 2A in (Crowther et al. 2003)]. Therefore, the drop in pH might have varied between participants, affecting the calculation of PCr recovery rates. We avoided this possible drawback by providing the participants with a real-time visual feedback on F_P relative to MVF_P. This way all our study participants sustained the target force of 85% MVF_P for 30 s. Finally, the discrepancy between our results and those of Crowther et al. (2003) might originate from sex-specific differences in the maximal rate of oxidative ATP synthesis.

Our findings that *cytochrome c oxidase* activity, capillary-to-fibre ratio, and MYH-1 fibre proportion of *vastus lateralis* and *soleus* muscles were not different between the two groups support our result that *in vivo* mitochondrial function was not impaired in women with type 1 diabetes, and lend further credence to the notion that oxidative capacity is not reduced in type 1 diabetes. In line with our results, other authors found no difference in *vastus lateralis* muscle *succinate dehydrogenase* activity (Fritzsche et al. 2008), skeletal muscle capillary-to-fibre ratio and fibre type distribution in *vastus lateralis* muscle (Wallberg-Henriksson et al. 1984) between patients with type 1 diabetes and healthy controls. However, there are also reports showing that MYH-1 fibre proportion in women and men with type 1 diabetes (Fritzsche et al. 2008) is reduced. Besides muscle fibre metabolism, oxidative capacity also depends on muscle capillarisation. In fact, the size of the capillary network determines mean red blood cell transit time and surface area for gas exchange and may also influence the exchange of substrates and metabolites and removal of heat (Saltin et al. 1986). However, capillary density relates more to submaximal (*e.g.* endurance capacity and ventilatory threshold) than to maximal measures (Saltin et al. 1968). In this study, neither capillarisation nor endurance capacity or ventilatory threshold were reduced in women with type 1 diabetes relative to healthy controls, indicating that submaximal aerobic exercise capacity was not impaired in women with type 1 diabetes.

It has been unclear up to now, whether maximal oxygen uptake and exercise performance (*i.e.* maximal power output during a GXT) are reduced in type 1 diabetes. While some authors reported that adolescents with type 1 diabetes display both reduced oxygen uptake (Gusso et al. 2008, Nadeau et al. 2010) and peak power (Nadeau et al. 2010) other investigators showed that these measures are unchanged in adults with type 1 diabetes (Fritzsche et al. 2008, Harmer et al. 2008, Veves et al.

1997). Here, we showed that neither maximal oxygen uptake (Fig. 2.3A) nor maximal power during a GXT was different between patients with type 1 diabetes and healthy controls. Furthermore, the similar maximal oxygen uptake between the two groups relied on similar perfusive (cardiac output, Fig. 2.3B) and diffusive (calculated arterio-venous oxygen difference, Fig. 2.3C) oxygen transport capacities, indicating that submaximal and maximal oxygen uptake, delivery and extraction are not reduced in women with type 1 diabetes.

It is well established that reduced insulin sensitivity is a prominent feature of type 2 diabetes. However, reduced insulin sensitivity can also be found in individuals with type 1 diabetes (DeFronzo et al. 1982, Harmer et al. 2008). We did not assess insulin sensitivity in this study and thus cannot quantify to which degree the type 1 diabetes participants were insulin resistant. Nevertheless, according to evidence indicating that type 1 diabetes patients are insulin resistant to some degree, and assuming that the type 1 individuals who participated in this study were not different in this regard, our data might be interpreted to support the suggestion of other scientists (Holloszy 2009) that insulin resistance is not the consequence of reduced oxidative capacity. Our finding that skeletal muscle mitochondrial capacity was not impaired in women with type 1 diabetes despite higher total and segmental fat masses (Tab. 2.1) supports this speculation, *i.e.* that reduced oxidative capacity is not instrumental to insulin resistance, especially in light of the negative correlation between both abdominal (Carey et al. 1996) and thigh fat (Goodpaster et al. 1997) and insulin sensitivity.

HbA1c levels above 7% are indicative of poor glycaemic control in type 1 diabetes (American Diabetes Association 2008). In this regard, it has recently been shown that endurance athletes with type 1 diabetes and high (> 7%) HbA1c have lower peak oxygen consumption, peak stroke volume, and peak cardiac output relative to athletes with low (< 7%) HbA1c (Baldi et al. 2010), and that HbA1c negatively correlates with peak stroke volume. Although we did not find a reduced mitochondrial capacity and aerobic exercise capacity in women with type 1 diabetes with a mean HbA1c of 7.6% (range 6.9-8.2%) relative to healthy female participants, linear regression revealed that women with type 1 diabetes exhibiting higher HbA1c have a lower k_{PCr} and peak cardiac output (Fig. 2.4). Taken together, these data might be interpreted to support the notion that in type 1 diabetes, oxidative capacity is correlated with glycaemic control, but that only at higher HbA1c levels (> 7.6%)

the impairment becomes significant relative to untrained healthy women.

We do acknowledge some limitations with the current investigation. For example, we calculated arterio-venous oxygen difference from the estimated oxygen uptake and cardiac output according to the Fick principle, instead of calculating arterio-venous oxygen difference and oxygen extraction from measured arterial and venous oxygen content and arterial oxygen concentration. A further limitation is that we did not directly estimate insulin sensitivity from euglycaemic hyperinsulinaemic clamps nor hepatic glucose production. However, the primary aim of this study was to determine the relationship between type 1 diabetes and oxidative capacity, irrespective of the degree of insulin resistance. We thus feel like our approach of combining non-invasive inert gas rebreathing with gas exchange measurements (to assess maximal oxygen uptake and cardiac output), to subsequently calculate stroke volume and arterio-venous oxygen difference according to the Fick principle, and to relate these systemic measures with *in vivo* maximal mitochondrial ATP synthesis rate and oxidative markers from muscle biopsies, provides novel information about the relationship between oxidative capacity and its underlying components in type 1 diabetes.

In summary, maximal oxygen uptake, maximal cardiac output and calculated arterio-venous oxygen difference, as well as ^{31}P-MRS-derived maximal rates of calf muscle oxidative ATP synthesis, *vastus lateralis* and *soleus* muscle fibre metabolic phenotype and capillarisation were not different between young women with type 1 diabetes and healthy women of similar age and activity level. Also, we found no difference in ventilatory threshold and endurance capacity between the two groups. However, in women with type 1 diabetes, glycaemic status affected oxidative capacity. We conclude that in young untrained women with type 1 diabetes and a mean HbA1c of 7.6% (range 6.9-8.2%), glycaemic status affects mitochondrial capacity and peak cardiac output, but that neither maximal nor submaximal aerobic capacity is reduced relative to healthy women of similar age and activity level.

Chapter 3

Electric pulse stimulation of cultured murine muscle cells reproduces gene expression changes of trained mouse muscle

Nathalie Burch[1,2], Anne-Sophie Arnold[1,3], Flurin Item[1,2], Serge Summermatter[1,3], Gesa Brochmann Santana Santos[3], Martine Christe[3], Urs Boutellier[1,2], Marco Toigo[1,2], and Christoph Handschin[1,3,4]

[1]Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland
[2]Exercise Physiology, Institute of Human Movement Sciences, ETH Zurich, Zurich, Switzerland
[3]Biozentrum, Department of Pharmacology/Neurobiology, University of Basel, Basel, Switzerland

PLoS One. (2010) 5:e10970

Introduction

Physical activity results in a number of phenotypic adaptations of skeletal muscle (Booth and Thomason 1991, Flueck 2006). If performed repeatedly, exercise thereby confers health benefits by preventing and ameliorating a number of chronic diseases, improving the quality of life and increasing life expectancy (Handschin and Spiegelman 2008). Intriguingly though, the molecular mechanisms that underlie the corresponding plastic changes of muscle fibres are poorly defined. Progress in understanding these mechanisms would be facilitated by an experimentally amenable cell culture model. Electrical and mechanical signals, *e.g.* motor neuron activity and muscle fibre stretch, are two of the major exercise-associated stimuli that result in a remodelling of muscle fibres. Motor neuron activation of muscle fibres can be replicated by electric pulse stimulation (EPS) of muscle myotubes in culture. For example, electrical stimulation of muscle cells in culture increases contractile properties (Thelen et al. 1997) and accelerates sarcomere assembly (Fujita et al. 2007). Furthermore, EPS-induced changes in gene expression patterns and metabolic properties have been reported (Nedachi et al. 2008, Park et al. 2008, Silveira et al. 2006). Unfortunately, the effects of electric stimulation on the development and function of cultured muscle cells remain controversial and vary between different muscle cell types and stimulation conditions (Naumann and Pette 1994, Pedrotty et al. 2005, Putman et al. 2000, Stern-Straeter et al. 2005).

To study exercise effects in an experimental cultured muscle fibre model, the gene expression changes triggered by exercise *in vivo* and the modulation of transcriptional activity of cultured, EPS-stimulated muscle cells have to be compared. Obviously though, *in vivo*, these changes differ dramatically between the acute response to a single bout of exercise and the chronic effects of training on muscle, with the latter conferring most of the health benefits (Booth et al. 2002, Booth and Lees 2007, Coffey and Hawley 2006). Despite the distinct outcome, some of the signalling pathways are conserved in both cases. For example, the expression of the peroxisome proliferator-activated receptor γ

coactivator 1α (PGC-1α) in humans and rodents is temporarily elevated after each exercise bout (Norrbom et al. 2004, Pilegaard et al. 2005, Terada et al. 2002), and exhibits a persistent basal elevation after chronic endurance training (Goto et al. 2000, Russell et al. 2003, Taylor et al. 2005) while maintaining additional inducibility with physical activity (Pilegaard et al. 2003, Coffey et al. 2006). The acute increase of PGC-1α levels and activity might primarily be responsible for increased oxidative metabolism and hence an elevated ATP synthesis (St-Pierre et al. 2003, Wright et al. 2007, Wu et al. 1999). Chronic elevation of PGC-1α is associated with a fibre-type switch from the glycolytic, fast-twitch type IIB and IIX fibres towards the oxidative, high-endurance type IIA and I muscle fibres (Handschin et al. 2007, Lin et al. 2002) as well as an increase in vascularisation (Arany et al. 2008). In fact, PGC-1α seems to regulate many, if not all of the adaptations of muscle fibres to chronic endurance training (Finck and Kelly 2006, Handschin and Spiegelman 2008, Handschin et al. 2007a, b, c, Handschin 2009, Lin et al. 2005) and leads to improved exercise performance as well as increased peak oxygen uptake (Calvo et al. 2008).

We aimed at establishing and validating an EPS condition that induces a gene expression pattern resembling that of a trained muscle. For that purpose, we tested EPS protocols in different muscle cell types, and because of the versatility and robustness of PGC-1α induction in the trained muscle (Vissing et al. 2005), we measured the expression of this gene as a prototypical exercise gene. Subsequently, the level of key genes in mitochondrial function, substrate uptake and oxidation were determined. The EPS-triggered changes of these genes in muscle fibres in culture were then compared to those in acutely exercised and trained mice, respectively. Our data suggest that the proper EPS conditions in muscle cells in culture qualitatively recapitulate some of the gene expression patterns observed in trained muscle.

Design and methods

Chemicals and cell culture reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS, calcium- and magnesium-free), 1x trypsin-EDTA, penicillin-streptomycin (P/S), and horse serum (HS) were purchased from Invitrogen (Basel, Switzerland). Bovine serum albumin (BSA) was obtained from Sigma (Buchs, Switzerland). SYBR-green was purchased from Sigma (Buchs, Switzerland). Trizol, reagents for DNase digestion and reverse transcription were obtained from Invitrogen (Basel, Switzerland). Real-time PCR primers were synthesised by Microsynth (Balgach, Switzerland).

Cell culture conditions

C2C12 (CRL-1772) and SOL8 (CRL-2174) cells were obtained from the American Type Culture Collection (ATCC) and cultured at 37°C in 5% CO_2 on 100-mm plastic dishes containing culture medium. These cell lines are standard mouse muscle cells that were obtained from glycolytic and oxidative muscles, respectively, and that are widely used as a cell culture model for muscle in culture. Culture medium consisted of DMEM supplemented with 1% P/S and 10% FBS for C2C12 and 20% for SOL8, respectively. For the experiments, the cells were distributed onto 6 well plates (Corning, New York, USA). Upon reaching about 90% of cell confluence, the culture medium was switched to differentiation medium consisting of DMEM supplemented with 1% penicillin/streptomycin and 2% horse serum. This differentiation medium was changed every 24 h. After 4-6 days, differentiation into myotubes was completed and the cells used for EPS.

Electric pulse stimulation (EPS)

EPS was applied to myotubes with a C-Pace EP culture pacer (IonOptix, Dublin, Ireland). This instrument emits bipolar pulses to the carbon electrodes of the C-dish with the electrodes hanging in the cell culture media. The contraction paradigm consisted of 1-s trains with 1-s pauses between the trains. During the 1-s trains, a 1-ms pulse stimulus with 14 V and a frequency of 50 Hz was applied. These 1-ms pulses most likely result in a combined activation of action potentials and the dihydropyridine receptor system (Cairns et al. 2007). The contractions of the myotubes were verified by examination under microscope. C2C12 and SOL8 cells were EPS treated either for 90 min ("acute" protocol), for 90 min each on 4 consecutive days at regular time intervals ("intermittent" protocol) or for 24 consecutive h ("continuous" protocol). Differentiation medium was exchanged 1 h before the stimulation was started. In order to test serum-free conditions, BSA medium (DMEM supplemented with 1% P/S and 0.1% BSA) was added to three wells of C2C12 cells instead of differentiation medium on the third and fourth day of stimulation or for the 24 h of stimulation. Cells were harvested in 1 ml Trizol 3 h after EPS similar to previous studies (Silveira et al. 2006).

Animal experiments

All procedures were approved by the Veterinary office of the canton of Zurich and performed following institutional guidelines. Male wild-type C57Bl/6J mice (age: 6 weeks, body weight: between 20 and 25 g) were maintained in cages with a 12 h:12 h light-dark cycle starting at 6:00 in a room at 22° C. Food and water were available *ad libitum*. The mice were randomly divided into experimental groups with seven (chronic) or eight (acute) mice per group. Exercise training was performed at the same time each day during the light cycle. At the end of the experiment, mice were sacrificed by CO_2 inhalation, blood was collected by cardiac puncture and death ensured by cervical dislocation.

Treadmill exercise

Mice were first familiarised with treadmill running for 3 days. The treadmill (Columbus Instruments, Ohio, USA) was equipped with an electric stimulation grid at the rear. The duration of these familiarisation runs was 5 min with a speed of 6 $m \cdot min^{-1}$ and an incline of +5°. On the day after familiarisation, body weights were measured and a forced incremental exercise test to exhaustion was performed. This test was started with an incline (α) of +5° and a speed of 6 $m \cdot min^{-1}$ for 5 min. After this initial phase, the speed was progressively increased by 2 $m \cdot min^{-1}$ every 3 min. Animals ran until exhaustion, which was defined as the inability to remain on the treadmill despite repeated contact with the electric grid (0.4 mA) and mechanical prodding for more than 10 s. Once exhaustion was reached, the power of the shock grid was turned off. Running time was measured and running distance, work and power were calculated. Distance is a function of time and speed (Distance = $v_{treadmill} \cdot t$). Work is the product of body mass, gravity, vertical speed and time (Work = $m_{mouse} \cdot g \cdot v_{treadmill} \cdot \tan[\alpha] \cdot t$). Power is calculated as the product of body mass, gravity and vertical speed (Power = $m_{mouse} \cdot g \cdot v_{treadmill} \cdot \tan[\alpha]$). The group that underwent training recovered for 2 days. Subsequently, these mice trained using the same treadmill protocol until 75% of the average distance of all the mice from the last exhaustion trial was reached. This training was performed on 4 days of the week, while the 5^{th} day was used for a new exhaustion test. This procedure (4 days training, 1 day exhaustion trial and 2 days of rest) was repeated for a total of 6 weeks when the gains in endurance parameters were significantly increased over those of control mice for two weeks in a row. The control group was kept at standard, sedentary conditions. All the mice were anesthetised with isofluran (Provet, Lyssach, Switzerland) either 4 h after the single performance trial for the acute exercise group or 3 days after their last endurance trial for the chronic exercise group. Animals were sacrificed by CO_2 inhalation, blood was collected by cardiac puncture before death was ensured by cervical dislocation.

Gene expression analysis and Western blots

Mice were dissected and muscle *gastrocnemius* was prepared. The frozen *gastrocnemius* was pulverised and RNA prepared using Trizol reagent according to the manufacturer's instructions (Invitrogen). C2C12 and SOL8 cells were directly lysed in Trizol reagent and subsequently processed. The purity of the resulting RNA was assessed by the 260 nm/280 nm ratio. Subsequently, DNase I digestion was performed and 1 μg of total RNA was reverse transcribed. Relative gene expression levels were determined by real time polymerase chain reaction using SYBR-green on an Mx3000P QPCR System (Stratagene, La Jolla, USA) according to the ΔΔCt method. Relative gene expression was normalised to 18S rRNA and TATA box binding protein (TBP) levels. The following primers were used: PGC-1α (forward TGA TGT GAA TGA CTT GGA TAC AGA CA, reverse GCT CAT TGT TGT ACT GGT TGG ATA TG), GABPA (forward CCA AGC ACA TTA CGA CCA TTT, reverse CCG TGG ACC AGC GTA TAG GA), TFAM (forward CCG AAG TGT TTT TCC AGC AT, reverse CAG GGC TGC AAT TTT CCT AA), ERRα (forward AGC AAG CCC CGA TGG A, reverse GAG AAG CCT GGG ATG CTC TT), Cyt c (forward GCA AGC ATA AGA CTG GAC CAA A, reverse TTG TTG GCA TCT GTG TAA GAG AAT C), ATPSyn (forward AGG CCC TTT GCC AAG CTT, reverse TTC TCC TTA GAT GCA GCA GAG TAC A), Ndufb5 (forward TTT TCT CAC GCG GAG CTT TC, reverse ATA AAG AAG GCT TGA CGA CAA ACA), COX5 (forward GCT GCA TCT GTG AAG AGG ACA AC, reverse CAG CTT GTA ATG GGT TCC ACA GT), MCAD (forward AAC ACT TAC TAT GCC TCG ATT GCA, reverse CCA TAG CCT CCG AAA ATC TGA A), Cpt1b (forward ATC ATG TAT CGC CGC AAA CT, reverse CCA TCT GGT AGG AGC ACA TGG), GLUT1 (forward CGA GGG ACA GCC GAT GTG, reverse TGC CGA CCC TCT TCT TTC AT), GLUT4 (forward GAT GAG AAA CGG AAG TTG GAG AGA, reverse GCA CCA CTG CGA TGA TCA GA), GYS1 (forward GAA CGC AGT GCT TTT CGA GG, reverse CCA GAT AGT AGT TGT CAC CCC AT), PDH (forward GAA GGC CCT GCA TTC AAC TTC, reverse ATA GGG ACA TCA GCA CCA GTG A), LDH (forward GGA AGG AGG TTC ACA AGC AG, reverse TCA CAA CAT CCG AGA TTC CA), MyHCI (forward CCT TGG CAC CAA TGT CCC GGC TC, reverse GAA GCG CAA TGC AGA GTC GGT G), MyHCIIa (forward ATG AGC TCC GAC GCC GAG, reverse TCT GTT AGC ATG AAC TGG TAG GCG), MyHCIIx (forward AAG GAG CAG GAC ACC AGC GCC CA, reverse ATC TCT TTG GTC ACT TTC CTG CT), MyHCIIb (forward GTG

ATT TCT CCT GTC ACC TCT C, reverse GGA GGA CCG CAA GAA CGT GCT GA), 18S rRNA (forward AGT CCC TGC CCT TTG TAC ACA, reverse CGA TCC GAG GGC CTC ACT A), TBP (forward GGC CTC TCA GAA GCA TCA CTA, reverse GCC AAG CCC TGA GCA AA). Western blots were performed on protein extracts from C2C12 cells. Antibodies were purchased from Santa Cruz (PGC-1α, MCAD) and Cell Signaling (Cycs, tubulin), respectively.

Lactate determination

Circulating lactate concentration in venous blood was measured 4 h and 3 days after exercise in the acute and the chronic exercise group, respectively. Lactate analysis was performed using a Biosen C_line (EKF-diagnostic, Barleben-Magdeburg, Germany).

Statistical analysis

Results are presented as mean while error bars depict standard deviation (SD). Unpaired, two-tailed student's t test was performed, statistical significance was defined as $P < 0.05$ and indicated by an asterisk. Eight mice per group, except for the trained cohort with seven animals, or three independent cell experiments were used.

Results

Electric pulse stimulation of C2C12 mouse muscle cells induces PGC-1α gene expression

Several different protocols for electric pulse stimulation (EPS) of muscle cells in culture have been proposed (*e.g.* see refs. [Fujita et al. 2007, Marotta et al. 2004,

Nedachi et al. 2008, 2009, Park et al. 2008, Silveira et al. 2006]). However, none of these approaches included a broad investigation of the transcriptional adaptations associated with an active muscle fibre. We thus tested two different EPS conditions and studied three different cell systems to establish stimulation conditions that recapitulate plastic changes on the gene expression level. As a marker for a trained muscle, we measured the relative expression of PGC-1α, a transcriptional coactivator that is regulated by endurance exercise in muscle and in turn controls many of the muscle adaptations to physical activity (Finck and Kelly 2006, Handschin 2009, Handschin and Spiegelman 2006, Lin et al. 2005). For that purpose, differentiated C2C12 myotubes were either stimulated for 90 min ("acute", Fig. 3.1A and D), for 90 min daily on 4 consecutive days ("intermittent", Fig. 3.1B and E) or for 24 h ("continuous", Fig. 3.1C). The cells were harvested 3 h after the EPS and the relative expression of PGC-1α was quantified by real-time PCR. A single stimulation for 90 min failed to alter PGC-1α expression significantly (Fig. 3.1A). In contrast, repeated EPS for 90 min daily on 4 consecutive days significantly induced PGC-1α gene expression (2.4 fold, Fig. 3.1B). Finally, a single EPS for 24 h resulted in the highest induction of PGC-1α transcript levels (2.9 fold, Fig. 3.1C). Silveira and colleagues (2006) demonstrated an enhanced reactive oxygen species (ROS) production in EPS-treated primary muscle cells and postulated an important role for elevated ROS levels in the induction of PGC-1α in these cells. We therefore studied if EPS of C2C12 cells in serum-free and hence antioxidant-reduced conditions exacerbates PGC-1α induction. However, elevation of PGC-1α gene transcription was lower using serum-free compared to standard differentiation media (Fig. 3.1D, 2.1 fold) or did not reach statistical significance (Fig. 3.1E). When stimulated for 24 h in serum-free conditions, C2C12 cells changed in morphology and adhesion and therefore, gene expression changes were not assessed for this condition. The discrepancies between the previously reported findings and our data could be explained by the different culture conditions and cell types that were used in the previous study (primary rat muscle cells, ref. [Silveira et al. 2006]) and in the present work (C2C12 muscle cells). To include additional mouse muscle culture models in our experiments, we tested the response of SOL8 cells, a myogenic cell line derived from mouse *soleus* primary cells, to the different EPS regimes and culture conditions. However, none of our stimulation protocols elicited a significant change in PGC-1α mRNA expression (data not shown). Whether this is due to the origin of SOL8 cells that were isolated from *soleus*, a muscle that shows a very modest response to endurance exercise, is unclear. Moreover, primary mouse muscle myotubes were overly sensitive to our

EPS conditions and did not survive the respective experiments (data not shown). As a consequence of these tests, we used C2C12 cells and the 24 h EPS paradigm in all subsequent experiments.

Improved exercise performance of mice after 6 weeks of endurance training

Health benefits of exercise are primarily associated with training whereas a single exercise bout is insufficient to induce the same effects. To be able to compare the transcriptional changes of stimulated C2C12 cells in culture to those in acutely exercised and trained muscle, we designed an exercise protocol for mice that iteratively adjusts the training load to the increased muscle function over 6 weeks (Fig. 3.2A). This trained mouse cohort was then compared to a group of mice that underwent one endurance trial (called “mice, acute exercise”) and to control mice that remained sedentary, respectively. The endurance trial and the training bouts consisted of treadmill running with incremental speed (Fig. 3.2B), either to complete exhaustion for the trials or to 75% of the distance of the last exhaustion test for the training. In order to achieve a clear distinction between the acute effects of exercise and the chronic adaptations, the acute group was sacrificed 4 h after the trial when blood lactate levels were still elevated (Fig. 3.2C). In contrast, the trained group was analysed 3 days after their last bout of exercise indicating an absence of confounding effects of the last acute exercise. At this time, the mice had normalised blood lactate (Fig. 3.2C). The improvement of endurance exercise capacity in the trained animals was documented by significant increases in time on the treadmill, distance, work and power after 6 weeks of training (Fig. 3.2D-G).

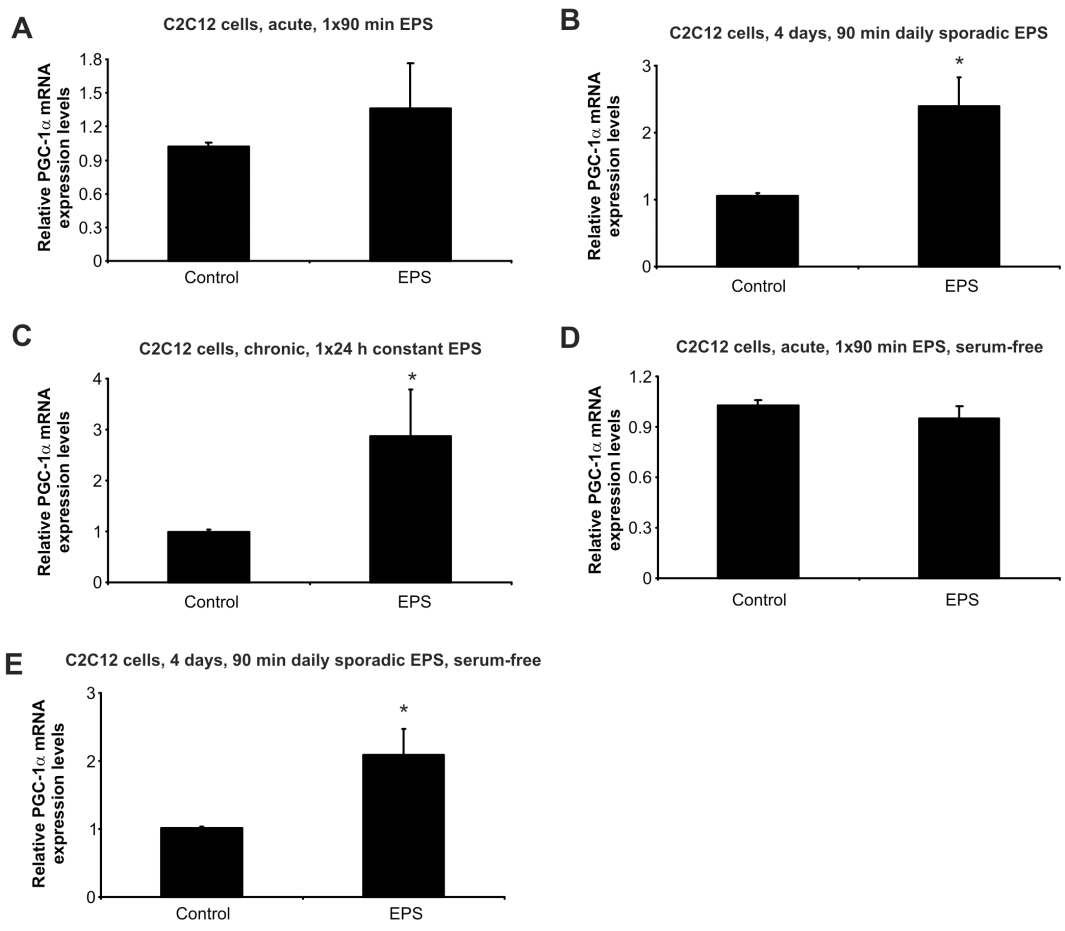

Figure 3.1: Electric pulse stimulation (EPS) of C2C12 myotubes induces PGC-1α gene expression. Differentiated C2C12 myotubes were electrically stimulated for 90 min (acute EPS, panels A and D), 4 consecutive days, 90 min each day (4 days, sporadic EPS, panels B and E) or for 24 h (24 h, chronic EPS, panel C). These experiments were either performed in regular differentiation medium (panels A, B and C) or in serum-free conditions with BSA (serum-free, panels D and E). Three hours after EPS, cells were harvested, RNA isolated and the relative expression of PGC-1α determined by real-time PCR. Bars are average levels of three independent experiments and error bars depict SD. $^{*}P < 0.05$. Abbreviations: EPS, electric pulse stimulation.

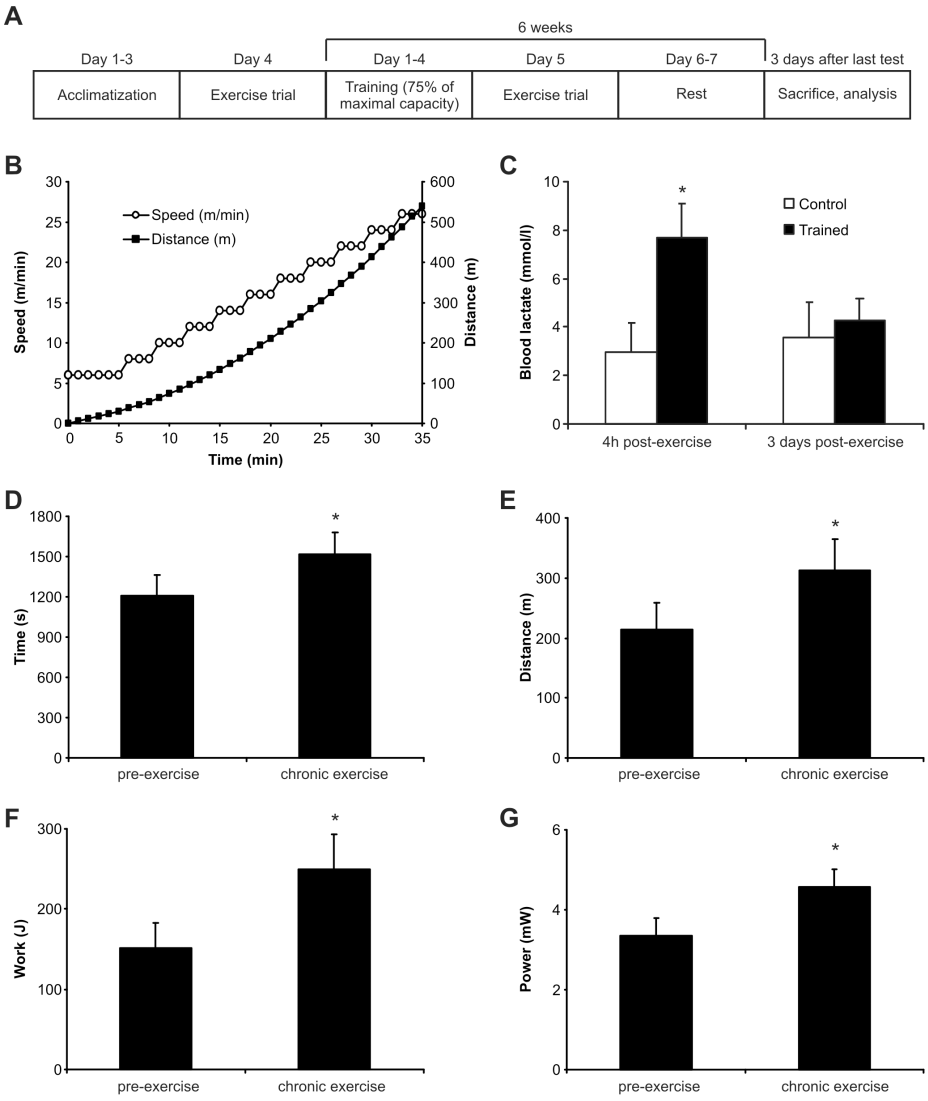

Figure 3.2: Endurance improvement of chronically exercised mice. A, After familiarisation, mice were trained for 6 weeks and sacrificed 3 days after the last bout of exercise. This cohort ("chronic exercise") was compared to a group of mice that performed one exercise trial ("acute exercise") and sedentary controls. B, An exercise protocol with incremental speed was chosen for the endurance trial and the daily training. C, Blood lactate measurement in mice after an acute bout of exercise (4 h post exercise) and after 3 days of recovery. D-G, Endurance running performance of mice before (pre-exercise) and after (chronic exercise) the 6 week training: running time (panel D), distance (panel E), work (panel F) and power (panel G). Bars are average levels and error bars depict SD. N= 8 animals per group pre-exercise and n=7 animals per group for the chronic exercise (one mouse died during the training period). *$P < 0.05$.

Induction of mitochondrial regulators and genes by EPS

Endurance training results in an improvement of mitochondrial function and oxidative metabolism (Chow et al. 2007, Coffey and Hawley 2007, Flueck 2006, Flueck and Hoppeler 2003, Hood et al. 2006). We thus tested how EPS of C2C12 cells affected some of the regulators of these processes and genes encoding oxidative phosphorylation (OXPHOS) proteins. In addition to PGC-1α, the GA-binding protein A (GABPA, alternatively called nuclear respiratory factor 2a or NRF2a), the mitochondrial transcription factor A (TFAM) and the estrogen-related receptor α (ERRα, NR3B1) were transcriptionally induced in stimulated cells compared to controls (2.9, 1.5, 1.5 and 1.8 fold, respectively, Fig. 3.3A). A similar expression pattern was observed in trained mice where PGC-1α was elevated 2.0 fold, GABPA 3.4 fold, TFAM 2.8 fold and ERRα 1.8 fold (Fig. 3.3B). In contrast, only PGC-1α transcription was significantly increased in acutely exercised animals (3.6 fold, Fig. 3.3C). ERRα, GABPA and TFAM regulate the transcription of nuclear- and mitochondrial-encoded mitochondrial genes, respectively (Scarpulla 2008). PGC-1α coactivates ERRα and GABPA and thereby greatly enhances the transcriptional activity of these proteins (Handschin et al. 2007, Huss et al. 2004, Mootha et al. 2004, Schreiber et al. 2004). Finally, the PGC-1α gene itself and those encoding these three transcription factors are transcriptional targets for the PGC-1α protein (Wu et al. 1999, Mootha et al. 2004, Handschin et al. 2003, Schreiber et al. 2003). As a consequence of this biological switch, OXPHOS and other mitochondrial genes are induced. Accordingly, EPS-treated C2C12 myotubes expressed higher levels of cytochrome c (Cyt c, 1.5 fold, Fig. 3.3D), ATP synthase subunit 5o (ATPSyn, 1.5 fold), NADH-ubiquinone oxidoreductase 1β subcomplex 5 (Ndufb5, 1.6 fold) and cytochrome c oxidase subunit 5b (COX5, 1.7 fold). In mice that were trained for 6 weeks, only cytochrome c and NADH-ubiquinone oxidoreductase 1β subcomplex 5 expression levels were significantly altered compared to sedentary control animals (3.0 and 2.0 fold, respectively, Fig. 3.3E). Four hours after an acute bout of exercise, none of these OXPHOS genes were significantly modulated in muscle (Fig. 3.3F).

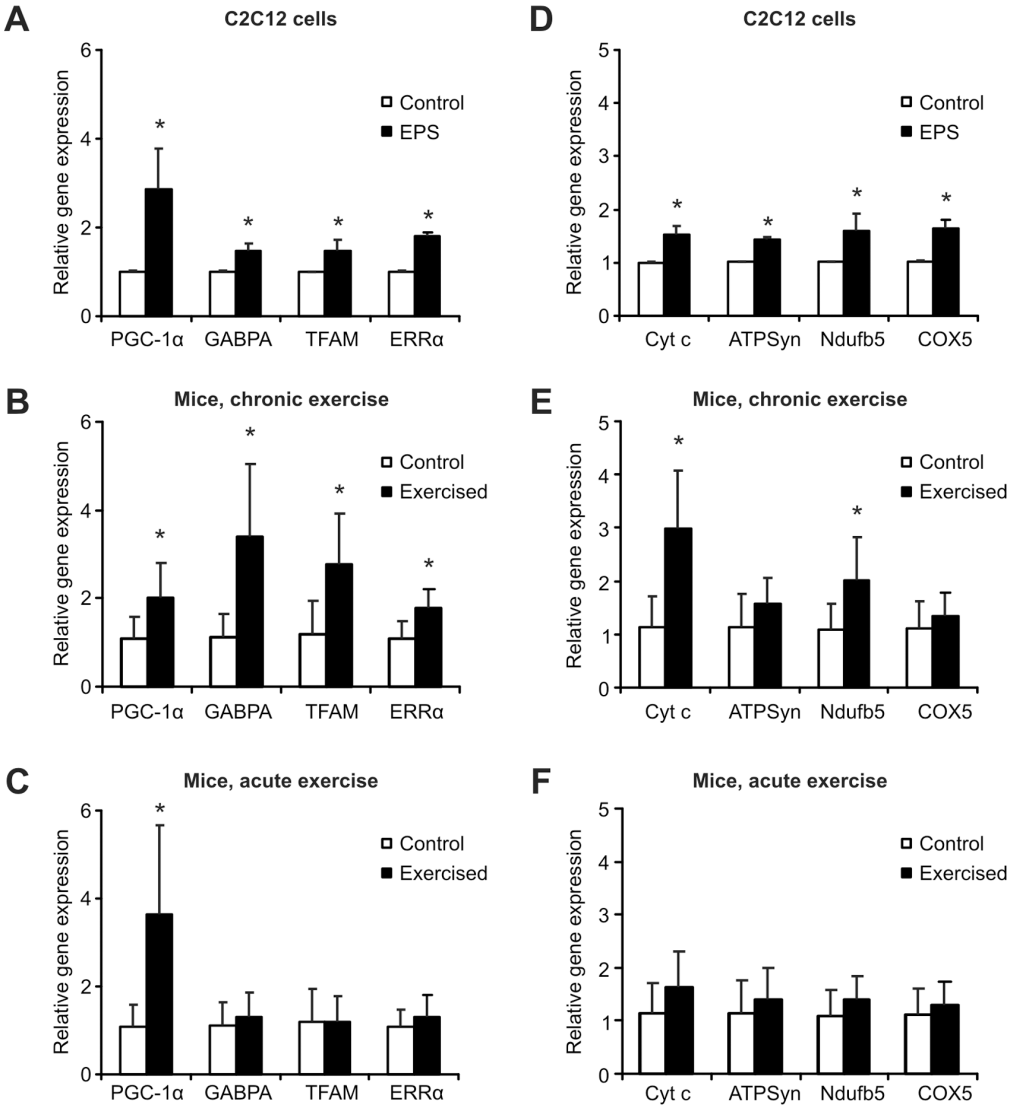

Figure 3.3: Electric pulse stimulation of C2C12 myotubes induces mitochondrial regulators and OXPHOS genes. A, D, C2C12 myotubes were EPS-stimulated for 24 consecutive hours. Three hours after stimulation, cells were harvested, RNA prepared and relative gene expression quantified by real-time PCR. The transcript levels were compared to unstimulated control cells. B, E, Mice were trained for 6 weeks. The animals were sacrificed 3 days after the last exercise bout, RNA isolated from *gastrocnemius* and relative gene expression determined by real-time PCR. Transcriptional induction was compared to the gene expression in sedentary control mice. C, F, Mice underwent one endurance trial and sacrificed 4 h later. RNA was isolated, relative gene expression quantified by real-time PCR and compared to that

of sedentary control mice. Bars are average levels and error bars depict SD. The data of the C2C12 study are from three independent experiments. In the animal studies, n= 8 animals per group were used except for the chronic exercise cohort with n=7. *P < 0.05. Abbreviations: PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; GABPA, GA-binding protein; TFAM, mitochondrial transcription factor A; ERRα, estrogen-related receptor α; Cyt c, cytochrome c; ATPSyn, ATP synthase subunit 5o; Ndufb5, NADH-ubiquinone oxidoreductase 1β subunit 5; COX5, cytochrome c oxidase subunit 5b; EPS, electric pulse stimulation.

Increased gene expression for substrate uptake and utilisation in exercised muscle cells and trained muscle

The increase in mitochondrial OXPHOS genes in endurance-trained muscle allows a greater oxidative metabolism of substrates, both of glucose and fatty acids (Holloway 2009, Corpeleijn et al. 2009). Accordingly, we studied some of the genes encoding for key enzymes in substrate import and usage. Fatty acid β-oxidation is primarily regulated by the import of fatty acids into mitochondria. This step is mediated by the carnitine-palmitoyltransferase 1b (Cpt1b) in muscle (Consitt et al. 2009, Holloway 2009). In the mitochondrial matrix, several proteins contribute to fatty acid oxidation, including the medium chain acyl-CoA dehydrogenase (MCAD) (Consitt et al. 2009, Holloway 2009). Cpt-1b and MCAD expression was elevated in EPS-treated C2C12 myotubes compared to untreated cells (Fig. 3.4A, 1.9 fold and 1.4 fold, respectively). A similar increase in MCAD and Cpt1b expression was observed in trained mice (Fig. 3.4B, 1.8 and 2.3 fold, respectively) whereas no change in transcription was seen in acutely exercised animals (Fig. 3.4C). These data indicate that EPS of muscle cells in culture triggers an increase in fatty acid β-oxidation resembling the adaptations of this pathway in chronic endurance exercise.

In addition to fatty acids, trained fibres import and use more glucose than untrained muscle fibres (Richter and Ruderman 2009, Santos et al. 2008). GLUT1 and GLUT4 are the major glucose transporters on the skeletal muscle cell membrane (Ebeling et al. 1998). In the myocyte, glucose is primarily stored in the form of glycogen or alternatively channelled into the glycolytic pathway (Ebeling et al. 1998).

Subsequent to the glycolytic conversion to pyruvate, glucose is either metabolised to acetyl-CoA by the pyruvate dehydrogenase and fed into the Krebs cycle in the mitochondria for oxidative metabolism or reduced to lactate under anaerobic conditions by the lactate dehydrogenase. In order to gain insights about glucose metabolism in EPS-treated cells, we measured the expression of GLUT1, GLUT4, glycogen synthase 1 (GYS1), pyruvate dehydrogenase (PDH) and lactate dehydrogenase (LDH). In stimulated myotubes, GLUT1 (2.2 fold), GLUT4 (3.2 fold), glycogen synthase 1 (1.6 fold) and pyruvate dehydrogenase (1.8 fold) transcription were significantly elevated (Fig. 3.4D). In contrast, lactate dehydrogenase expression was unchanged (Fig. 3.4D). This gene expression pattern resembles that of trained mice where GLUT1 (3.0 fold), glycogen synthase 1 (3.6 fold) and pyruvate dehydrogenase (2.3 fold) were significantly induced (Fig. 3.4E). GLUT4 and lactate dehydrogenase gene expression changes failed to reach statistical significance (Fig. 3.4E). In contrast, none of the transcripts were altered in acutely exercised animals (Fig. 3.4F). In summary, our findings imply an increased import and oxidative metabolism of glucose as well as an elevated glycogen synthesis in EPS-treated C2C12 myotubes similar to the changes in trained muscle.

Altered expression of myosin heavy chain isoforms in stimulated muscle cells

Skeletal muscle plasticity following endurance exercise extends beyond metabolic gene expression. For example, a close association between oxidative capacity and the levels of the myosin heavy chains I and IIA has been reported (Pette and Staron 2000, 2001, Staron 1997). These oxidative, high endurance muscle fibres are classified as type I and IIA fibres (Pette and Staron 2000, 2001, Staron 1997). In contrast, the type IIX and IIB muscle fibres are characterised by a fast twitching that generates a high force and express the myosin heavy chain isoforms IIX and IIB (Pette and Staron 2000, 2001, Staron 1997). We studied if our EPS protocol can induce expression changes of genes other than those that are directly involved in substrate metabolism and therefore quantified the relative transcript levels of the four main myosin heavy chain isoforms expressed in the adult muscle. These experiments revealed a significant elevation of the expression of myosin heavy chain I (MyHCI, 1.7 fold) and myosin heavy chain IIX (MyHCIIx, 2.0 fold), whereas

transcription of myosin heavy chain IIA and IIB (MyHCIIa and MyHCIIb) was unchanged (Fig. 3.5A). Our chronic exercise paradigm elicited similar changes *in vivo*. In these mice, myosin heavy chains I and IIX transcript levels were upregulated (2.4 and 1.9 fold, respectively) while the expression of myosin heavy chains IIA and IIB was unchanged (Fig. 5B). In muscle tissue of acute exercise mice, none of the myosin heavy chains exhibited altered expression levels (Fig. 3.5C). Thus, EPS of C2C12 myotubes triggered a modulation of myofibrillar gene expression resembling that of trained muscle.

Changes in protein levels in EPS-treated C2C12 myotubes

The relative changes in transcript levels and those observed for the respective protein can differ significantly, Thus, to test whether the EPS treatment of C2C12 myotubes also alters protein levels in addition to the induction in gene expression, we performed Western blots using antibodies for a selection of the genes that were analysed by real-time PCR (Fig. 3.6). We observed higher protein levels for PGC-1α, cytochrome c (Cycs) and the medium chain acyl-CoA dehydrogenase (MCAD) (Fig. 3.6) that reflect the higher transcript levels of the respective genes (Fig. 3.3A, 3.3D and 3.4A, respectively).

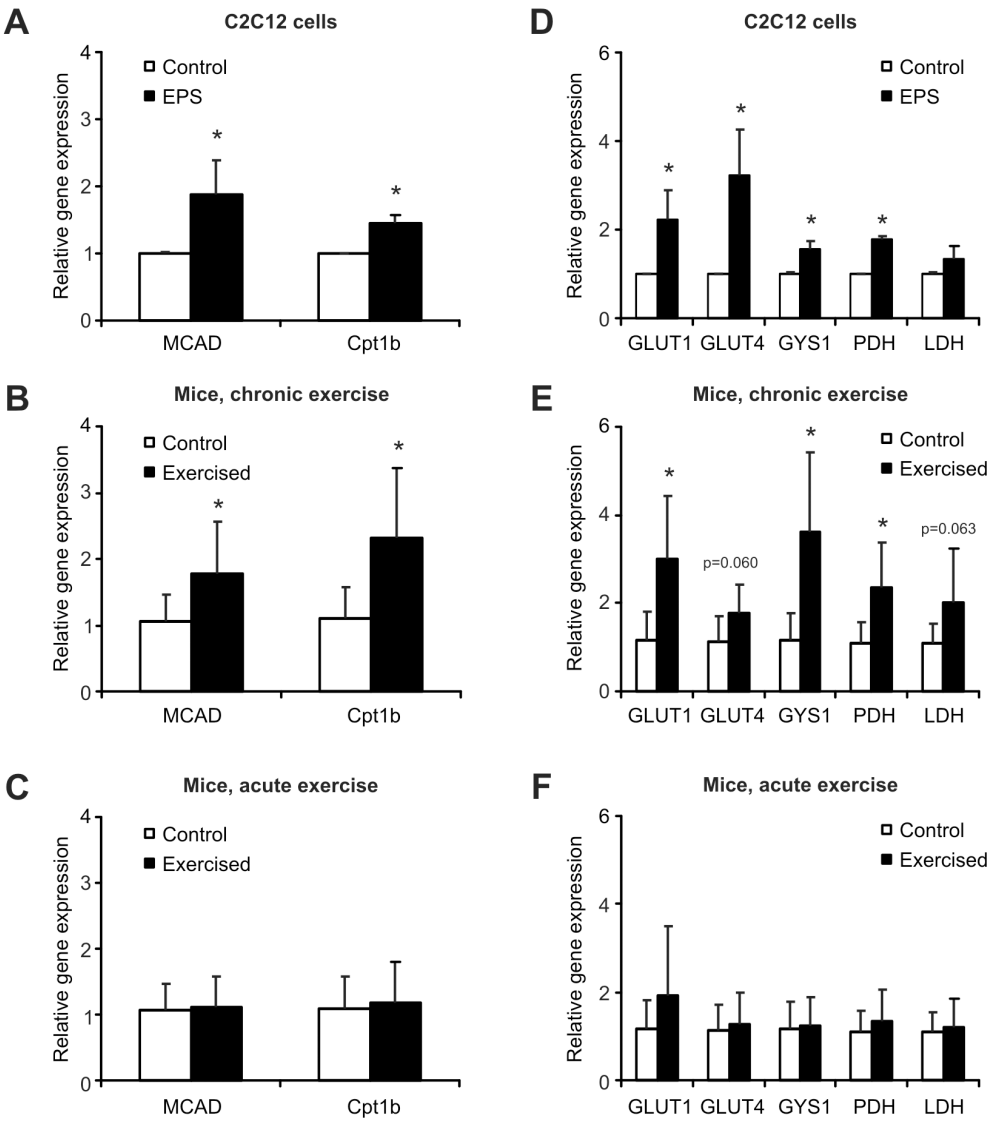

Figure 3.4: Electric pulse stimulation of C2C12 myotubes modulates fatty acid and glucose metabolism. A, D, C2C12 myotubes were EPS-stimulated for 24 consecutive hours. Three hours after stimulation, cells were harvested, RNA prepared and relative gene expression quantified by real-time PCR. The transcript levels were compared to unstimulated control cells. B, E, Mice were trained for 6 weeks. The animals were sacrificed 3 days after the last exercise bout, RNA isolated from gastrocnemius and relative gene expression determined by real-time PCR. Transcriptional induction was compared to the gene expression in sedentary control mice. C, F, Mice underwent one endurance trial and sacrificed 4 h later. RNA was isolated, relative gene expression quantified by real-time PCR and compared to that of sedentary control mice. Bars are average levels and error bars depict SD. The data

of the C2C12 study are from three independent experiments. In the animal studies, n= 8 animals per group were used except for the chronic exercise cohort with n=7. *P < 0.05. Abbreviations: MCAD, medium chain acyl-CoA dehydrogenase; Cpt1b, carnitine palmitoyltransferase 1b; GLUT1, glucose transporter 1; GLUT4, glucose transporter 4; GYS1, glycogen synthase 1; PDH, pyruvate dehydrogenase; LDH, lactate dehydrogenase; EPS, electric pulse stimulation.

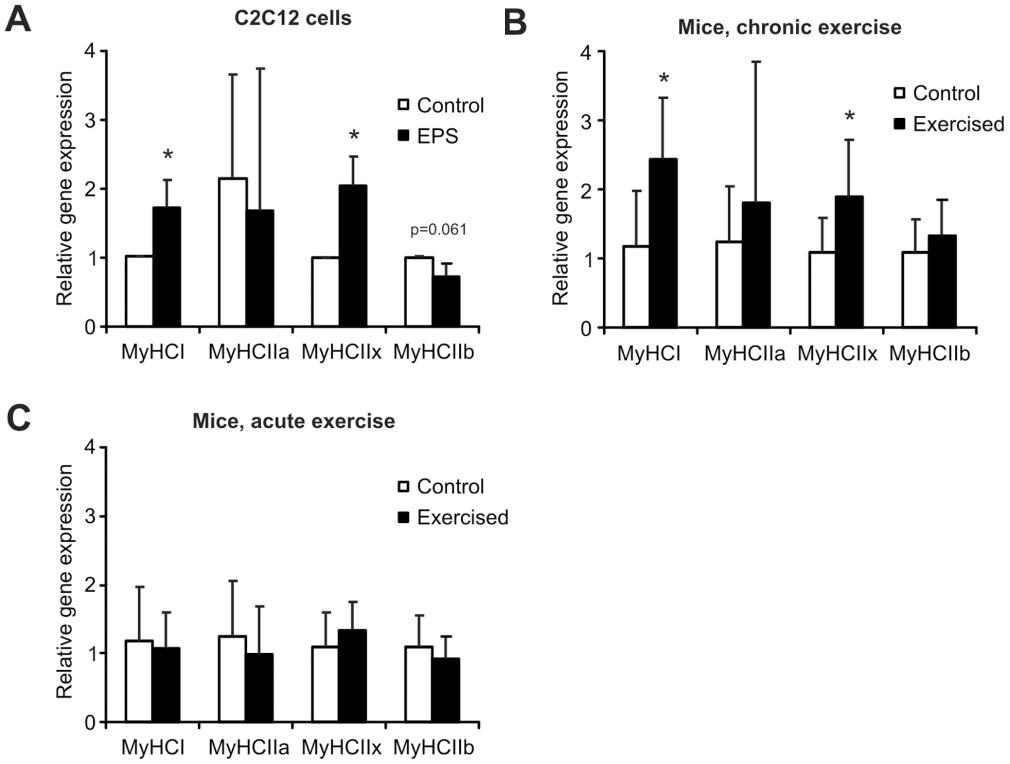

Figure 3.5: Electric pulse stimulation of C2C12 myotubes alters the myosin heavy chain expression patterns. A, C2C12 myotubes were EPS-stimulated for 24 consecutive hours. Three hours after stimulation, cells were harvested, RNA prepared and relative gene expression quantified by real-time PCR. The transcript levels were compared to unstimulated control cells. B, Mice were trained for 6 weeks. The animals were sacrificed 3 days after the last exercise bout, RNA isolated from *gastrocnemius* and relative gene expression determined by real-time PCR. Transcriptional induction was compared to the gene expression in sedentary control mice. C, Mice underwent one endurance trial and sacrificed 4 h later. RNA was

isolated, relative gene expression quantified by real-time PCR and compared to that of sedentary control mice. Bars are average levels and error bars depict SD. The data of the C2C12 study are from three independent experiments. In the animal studies, n= 8 animals per group were used except for the chronic exercise cohort with n=7. *P < 0.05. Abbreviations: MyHCI, myosin heavy chain I; MyHCIIa, myosin heavy chain IIa; MyHCIIx, myosin heavy chain IIx; MyHCIIb, myosin heavy chain IIb; EPS, electric pulse stimulation.

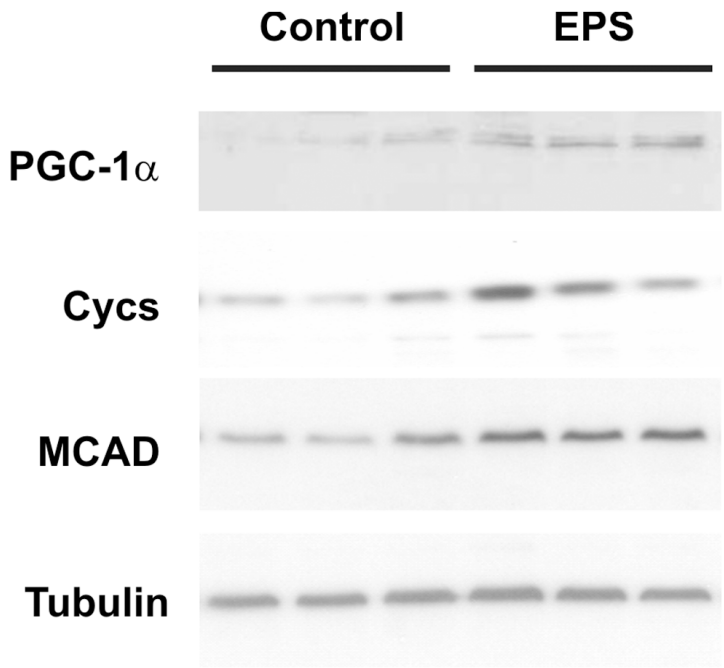

Figure 3.6: Elevation of protein levels in EPS-treated C2C12 myotubes. Protein extracts of control and EPS-treated C2C12 myotubes were analyzed in Western blots for the expression of PGC-1α, cytochrome c (Cycs), medium chain acyl-CoA dehydrogenase (MCAD) and tubulin.

Discussion

Exercise results in extensive adaptations in skeletal muscle and other organs (Booth and Lees 2007, Flueck and Hoppeler 2003, Handschin and Spiegelman 2008). Due to this complexity, our understanding of the molecular processes in these plastic changes is rudimentary. Furthermore, experimentally amenable model systems for exercise remain elusive. In this report, we describe how a cell culture model can be established to study at least some of the gene expression changes in the trained muscle. We found that key genes in metabolic processes, substrate uptake and oxidation are regulated by EPS in these cells. The pattern of gene expression qualitatively resembles very closely that of trained, but not acutely exercised muscle. Importantly, adaptation of muscle to training confers health benefits much more than the acute changes following a single bout of exercise.

Our analysis centered on the expression and function of the coactivator PGC-1α. This gene is central to the adaptations of muscle to exercise by transcriptionally regulating this biological programme. Upon ectopic expression with viral vectors, PGC-1α induces and coactivates some of its binding partners (Mootha et al. 2004). Here, we demonstrate that EPS-triggered elevation of PGC-1α is likewise sufficient to increase the levels of these transcription factors and subsequently, of mitochondrial OXPHOS genes. Moreover, the relative expression levels of genes encoding fatty acid import and β-oxidation proteins and those involved in glucose uptake and conversion of pyruvate to acetyl-CoA suggest that concomitant with an elevated mitochondrial function, substrate availability and metabolism are increased in EPS-treated C2C12 cells and trained muscle. Finally, the elevated expression of the glycogen synthase in EPS-treated cells mirrors that of glycogen synthase in exercise *in vivo*. In the latter, augmented glycogen synthesis helps to replenish and expand the glycogen storage in trained muscle (Ivy 1991, Nielsen and Richter 2003). Thus, the gene expression pattern in EPS-treated C2C12 myotubes suggests an extensive metabolic remodelling as found in trained muscle.

While the metabolic gene expression determines substrate usage, other muscle fibre parameters, such as fibre contraction speed and peak force generation, are controlled by myofibrillar proteins. The respective gene expression of the myosin heavy chain isoforms is used to classify the fibres into different fibre-types (Spangenburg and Booth 2003). The relative proportion of these fibre-types is to a large extent regulated by the motor neuron input (Thomas and Ranatunga 1993), although additional factors including genetic predisposition and imprinting exist (Hoh 1991, Huang et al. 2006, Partridge 2005). The lack of innervation of muscle cells in culture is likely to contribute to the poorly attributable fibre-type of many muscle cell lines. Electric stimulation can at least partially substitute for the missing motor neurons. We have observed an increase in the expression of specific myosin heavy chains in EPS-treated C2C12 myotubes. Our data suggest that with an adjusted protocol, *e.g*. slow, continuous stimulation or fast, sporadic stimulation, certain fibre-types can be promoted in these cells in culture. Moreover, in combination with other approaches such as addition of slow or fast muscle extracts (Matsuoka and Inoue 2008) or continuous mild heat stress (Yamaguchi et al. 2010), EPS of muscle cells in culture might be used to study fibre-type-specific properties.

Some of the previous studies on EPS in muscle cells in culture indicated that serum-free conditions might be better in inducing exercise-like adaptations compared to cells that are cultured with serum (Silveira et al. 2006). It is possible that the elevated levels in reactive oxygen species in serum-free medium contributes to EPS-mediated induction of muscle gene expression (Silveira et al. 2006). Interestingly, a similar negative effect of administration of large doses of antioxidant vitamins on exercise performance in humans has been shown (Ristow et al. 2009). In our experiments, no significant difference between serum-containing and serum-free conditions was observed, which could be explained by differences in culture and EPS conditions. Nevertheless, these findings indicate that EPS-treated C2C12 cells might be used to study the effects of reactive oxygen species and antioxidants, respectively, on muscle in exercise. Moreover, it might be interesting to use a known composite serum for these experiments, which then can be supplemented with additional factors and study their effect on exercise-like adaptation.

In summary, we have established an experimental protocol to qualitatively simulate some of the exercise-induced changes in gene expression in muscle cells in culture, at least for the genes that were included in the study. Clearly, a single bout of

stimulation of cell in culture does not reflect the complexity and time-dependent adaptation of skeletal muscle to exercise *in vivo*. Besides the missing neuronal activation, other stimuli of training adaptation, *e.g*. the hormonal milieu, are missing. Second, the determination of protein levels, enzymatic activates and functional read-outs might differ between the cells in culture and muscle *in vivo*. We tested some of the proteins for which increased gene expression was observed in the EPS-treatment and found elevated protein levels for PGC-1α, Cycs and MCAD (Fig. 3.6). In any case, such an experimental system allows the delineation of molecular mechanisms important for the contracting muscle fibre that cannot be performed in muscle *in vivo*. In the future, this model system will have to be adapted and refined. In particular, combining EPS with mechanical stretch or temporary hypoxia might further help to approximate the environment that a fibre of a trained muscle is exposed to. Similarly, co-cultures of motor neurons with these muscle cells might improve the comparability of the culture systems with muscle *in vivo*. These interventions might increase the activation of the different signalling pathways that converge on PGC-1α in the exercised muscle (Handschin 2010) and thereby further improve this cell culture model system. This will be tested by quantifying the activation of these pathways, *e.g*., phosphorylation of the AMP-dependent protein kinase and its cellular targets. Ultimately, these cells might constitute a key tool to further elucidate the molecular mechanisms of muscle adaptation to activity and inactivity and test novel approaches to treat fibre atrophy and muscular dystrophies.

Chapter 4

Adaptations to simultaneous vibration and resistance exercise with vascular occlusion

Marco Toigo[1,2,3], Flurin Item[1,2], Jonas Denkinger[1], Piero Fontana[1,3], Markus Weber[4], and Urs Boutellier[1,2]

[1] Exercise Physiology, Institute of Human Movement Sciences and Sport, ETH Zurich, Zurich, Switzerland
[2] Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland
[3] exersciences gmbh, Zurich, Switzerland
[4] Department of Visceral and Transplantation Surgery, University Hospital Zurich, Zurich, Switzerland

Submitted

Introduction

Exercise is a general term used to describe activities involving generation of force by the activated muscle(s) that results in disruption of a homeostatic state (Winter and Fowler 2009). In the traditional view exercise can be classified as either "resistance" or "endurance". In humans, the outcomes of resistance exercise include increases in maximal muscle power, skeletal muscle mass, and muscle fibre cross-sectional area (CSA) (Tesch 1988) with little or no increases in maximal oxygen consumption (Gettman et al. 1978) or the overall capillary-to-fibre ratio (Campos et al. 2002, Tesch 1988). Conversely, endurance exercise leads to increases in maximal oxygen consumption, maximal cardiac output, and time to exhaustion at submaximal power (Holloszy and Coyle 1984). Endurance exercise also increases muscle mitochondrial biogenesis, oxidative enzymes, and capillarisation. However, it does not increase maximal muscle power or mediate muscle hypertrophy (Holloszy and Coyle 1984). There are also adaptations that are common to both exercise modalities, namely the directional changes in myosin heavy chain (MYH) fibre type expression after prolonged training from 2X to 2A (Spangenburg and Booth 2003).

Whole body vibration exercise is a recent exercise modality that is largely unknown to the scientific community relative to classical strength and endurance exercise regimens, despite its popularity amongst exercisers and patients. The literature suggests that for sports training vibration exercise is an effective method to increase jump height in a shorter time than conventional resistance exercise (Rittweger 2010). The increase in jump height is correlated with an increase in maximal knee extension or jumping power, and this increase in maximal power is correlated with an increase in muscle mass (Rittweger 2010). These effects can be potentiated when resistance exercise is added on top of vibration exercise (Rittweger 2010).

Notably, resistance and endurance exercise cannot be performed simultaneously (*i.e.* on top of each other), but instead each exercise modality has been supplemented with superimposed blood flow restriction to potentiate its training

effects and broaden its fields of application. The combination of low-load [20-50% 1-repetition maximum (1-RM)] resistance exercise with blood flow restriction results in gains in torque and hypertrophy that are commensurate with traditional high-load (> 70% 1-RM) exercise (Burgomaster et al. 2003, Takarada et al. 2002, Takarada et al. 2000). This process also promotes fatigue resistance, as measured by an increased end-test torque after 50 maximal isokinetic leg extensions (Takarada et al. 2002). On the other hand, 1-legged cycling exercise (45 min per session, 4 sessions per week for 4 weeks) with 50 mmHg (6.67 kPa) external pressure over the working leg can increase oxidative enzyme activity, capillarisation, maximal oxygen uptake, endurance capacity, and glycogen storage compared with non-ischemic training of same duration and power output (Sundberg 1994). Furthermore, this type of training reduces maximal isokinetic (ω = 3.14 rad·s^{-1}) torque, tends to increase the proportion of MYH-1 fibres, and reduces the proportion of MYH-2X fibres (Sundberg 1994).

Taken together, the simultaneous combination of vibration exercise and resistance exercise enhances maximal power, whereas the simultaneous combination of resistance exercise and vascular occlusion increases fatigue resistance (*i.e.* time to exhaustion at submaximal power). An unaddressed question is whether the simultaneous combination of whole body vibration, resistance exercise and restricted blood flow (subsequently termed VRO) will facilitate a concurrent increase in maximal (*e.g.* during jumping) and submaximal (*e.g.* during cycling) measures of human power output and fatigue resistance.

The goal of this study was to determine how a 5-week VRO training regime influenced the mean response in maximal jumping power, maximal isokinetic (ω = 3.14 rad·s^{-1}) leg extension power, maximal cycling power, time to exhaustion at submaximal (85%) cycling power, and end-test torque following 50 maximal concentric isokinetic leg extensions in young women. Also myocellular and cardio-circulatory adaptations were assessed to relate functional and cellular/organic adaptations. A control group of young women without exercise were measured over the same period to account for any systematic biases in measurements that might occur during the acquisition of muscle tissues, tissue processing, body composition assessment, and functional testing.

Materials and Methods

Participants

We recruited 21 sedentary young women by placard and randomly assigned them to two groups (vibration exercise + resistance exercise + vascular occlusion [VRO]: 12 women; no training [CON]: 9 women). Participants in the VRO group were 23.5 (3.0) years old. Their body mass index was 21.6 (2.6) $kg \cdot m^{-2}$, and maximal oxygen consumption was 2.21 (0.25) $l \cdot min^{-1}$. Participants in the CON group were 24.5 (SD 3.8) years old and had a body mass index of 21.8 (1.7) $kg \cdot m^{-2}$, and their maximal oxygen consumption was 2.21 (0.48) $l \cdot min^{-1}$. These characteristics were not different between groups. All study participants were healthy. Nine (VRO) and seven (CON) women were taking oral contraceptives. After completing a routine health questionnaire, the participants were informed about the procedures applied and about the associated risks. The participants then signed an informed consent. All experiments were approved by the ethics committee of the canton of Zurich, and the study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki for human experimentation.

Study design and exercise protocol

The study consisted of 1) pretests; 2) 6 weeks of VRO or a similar period without exercise for CON (5 weeks with progressively increasing training load, last week with unchanged load); and 3) posttests (Fig. 4.1). This time frame was chosen because substantial and significant gains in muscle power are consistently achieved with vibration training intervals of 6 weeks, after which there may not be any further improvement (Rittweger 2010). The employed exercise regimen is described according to our previous recommendations (Toigo and Boutellier 2006). During the study, macronutrient intake was not monitored, and participants were not given

any food or dietary supplements.

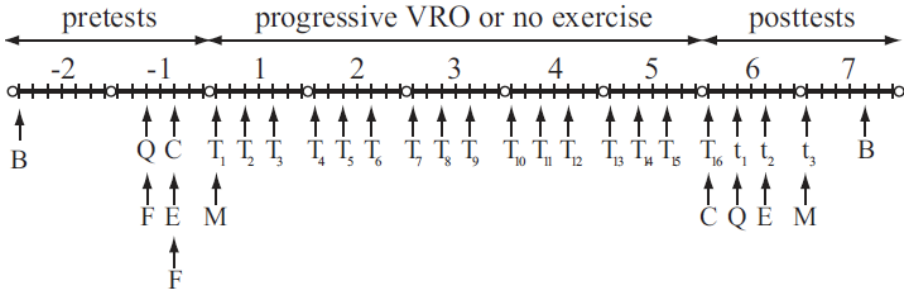

Figure 4.1: Overview of experimental protocol on a weekly scale (-2 to 7). B, biopsy; F, familiarisation session with respect to training and testing; Q, graded cycling exercise test; E, constant-load exercise test; C, dual-energy X-ray absorptiometry; M, muscle testing (mechanography and isokinetic dynamometry); $T_{1\text{-}16}$, training sessions with progressive training load; $t_{1\text{-}3}$, training sessions with constant (same as during T_{16}) training intensity. VRO, Galileo® vibration + resistance exercise + vascular occlusion.

Pre- and posttests. Before and after VRO we performed baseline measurements to assess maximal oxygen consumption, maximal cardiac output, cycling endurance capacity, isokinetic leg extension fatigue resistance, training load, thigh lean mass, as well as *vastus lateralis* muscle fibre size, fibre phenotype and capillarisation. Before the pretests, participants could familiarise themselves with the training and testing procedures on two separate occasions.

Exercise regimen. Participants of the VRO group performed three supervised progressive training sessions per week on alternate days (*i.e.* Monday, Wednesday, Friday) for 5 weeks (16 training sessions), followed by three training sessions with the same load and time under tension (TUT) as during the last progressive training session (Fig. 4.1). At the beginning of each exercise session, participants performed a warm-up (3 min), which involved squat and upright heel rise movements on a Galileo® side-alternating vibration plate (Novotec, Pforzheim, Germany) with vibration frequencies ranging from 6-30 Hz using various vibration amplitudes. The warm-up was followed by six sets of VRO: loaded (Multipower®, Technogym,

Gambettola, Italy) squats (three sets) and heel rises (alternating between two sets of upright heel rises and one set of seated heel rises or vice versa) were performed while standing on the Galileo® (Novotec, Pforzheim, Germany) oscillating at 30 Hz. A frequency of 30 Hz has been shown to be the optimal frequency for producing the greatest magnitude of response in EMG activity of *vastus lateralis* muscle in an isometric half-squat position (Cardinale and Lim 2003). Feet were placed on the center of the plate with the calcanei 0.28 (0.03) m apart during squats, as well as 0.25 (0.03) and 0.19 (0.03) m apart during upright and seated heel rises, respectively, with toes pointing slightly outwards. The corresponding vibration amplitudes were ~$3{\cdot}10^{-3}$ m ($6{\cdot}10^{-3}$ m from top to bottom) for squats and ~$2{\cdot}10^{-3}$ m ($4{\cdot}10^{-3}$ m from top to bottom) for heel rises. Participants wore skid-proof socks to eliminate any damping of the vibration which could have been caused by footwear.

For both, squat and heel rise exercises (three sets each), a duty cycle of 4 min on (maximal tolerable limit) and 1 min off was employed. Participants rested for 5 min between squats and heel rises. Three sets per muscle group were chosen in order to attain occlusion times (*i.e.* 12 min per session) that were in-between of those previously published (Pierce et al. 2006, Reeves et al. 2006). Vascular occlusion during the on phase of the duty cycles was induced by inflating tourniquet cuffs (thigh: width 0.09 m, length 0.76 m; calf: width 0.075 m, length 0.46 m; VBM, Sulz a.N., Germany) affixed to the inguinal fold region of the thigh, or to the proximal portion of the lower leg, centered in the space between the superior aspect of the *gastrocnemius* muscle and the inferior edge of the patella to suprasystolic pressure, *i.e.* 197 (3) mmHg [26.26 (0.40) kPa]. The suprasystolic pressure employed here was the highest pressure that was tolerated by the participants for the duration of the on phase of the duty cycles (4 min each). For both, squat and heel rise exercises, tourniquet cuffs were inflated right before the first set and remained inflated until the 4 min of vascular occlusion were complete. The cuffs were then deflated to a pressure of 100 mmHg (13.33 kPa), and the participants rested for 1 min before the cuffs were quickly reinflated for the subsequent set. Right upon cuff inflation, Galileo® vibration was initiated in parallel with squat or heel rise exercise.

Load magnitude was adjusted from prior knowledge (familiarisation sessions) in order to induce volitional failure within 40-60 s of exercise. Loads were adjusted progressively during the training period, *i.e.* in every training session either TUT or load magnitude were increased (2.5-5.0 kg). Load magnitudes (including the 4.5 kg

barbell and the plate loading mass) during the first training session corresponded to 57.5 (16.0) and 61.7 (21.1) % body mass for the first set of squat and upright heel rise exercise, respectively. For squat sets two and three the loads were reduced by 10.0 (4.0) and 15.9 (6.5) % body mass. Load reduction was 6.8 (4.1) % body mass for heel rise sets two and three. At the onset of squat exercise, participants grasped the loaded barbell, which was locked on the supports at shoulder height, with their hands spaced slightly more than shoulder width apart. Subsequently, they released the barbell with a twist of their wrists, positioned it on the back of their necks, and initiated squatting by bending their knees and flexing the hip.

One repetition comprised squatting over the individual's maximal range of motion (ROM), usually from near complete extension to a knee angle of slightly less than 90° in 4 s (eccentric action) and back to near complete extension in 4 s (concentric action). Transition from eccentric to concentric action was performed smoothly within 1-2 s. Thus, TUT for one repetition was 9-10 s, allowing four to six repetitions per set. Squat ROM in terms of vertical displacement of the barbell along the guide rod of the barbell support was 0.72 (0.05) m. Volitional exhaustion (task failure) was defined as the point in time where the participants failed to extend their legs. Safety stops along the guide rod were set to allow for safe and full range exercise to volitional exhaustion. Participants were advised to breathe as smoothly as possible. Exercise form and functional anatomy were always controlled by the same training supervisor, and whenever necessary the participants were corrected in real time. At task failure, the training supervisor stopped vibration and helped the participants to adopt an upright posture. Subsequently, the participants stood still on both feet (body mass symmetrically distributed) for 3 min while the inflated tourniquet cuffs were still on. Afterwards, cuffs were deflated to a pressure of 100 mmHg (13.33 kPa) for 1 min before they were reinflated for the next set. Heel rise exercise was performed analogous to squat exercise.

Experimental procedures

Muscle biopsy analyses. During the pre- and posttesting period of time, we obtained a percutaneous biopsy [average tissue mass per biopsy: 20.3 (3.1) mg] after local anaesthesia with 1% lidocaine from the middle portion of the nondominant *vastus*

lateralis muscle, using a ProMag Ultra device and 14 gauge needles (Angiotech Pharmaceuticals, Gainesville, FL, USA), as previously described (Walti et al. 2006)). After removal, the muscle tissue was immediately mounted in an embedding medium (Tissue-Tek®, Sakura, Zoeterwoude, The Netherlands), snap frozen in isopentane cooled to −160 °C with liquid nitrogen, and subsequently stored at −80 °C until use. Consecutive 12 µm sections were cut on a microtome at −25 °C and mounted on glass cover slides for further histochemical and immunohistochemical analyses. For all fibre analyses, only fibres fully encircled by adjacent fibres were evaluated. Measurements were made for at least 50 of each of the main fibre types (*i.e.* MYH isoform type 1 and 2). Previous studies investigating the skeletal muscle fibre sample size required for a reliable, valid representation of an individual's average fibre area and capillary-to-fibre ratio showed that 50 fibre measurements per individual for type 1 and 2 fibres and capillary contacts are sufficient to characterise type 1 and 2 fibre areas and capillary-to-fibre ratio of an individual (McCall et al. 1998, Porter et al. 2002). We stained the serial cryocut cross-sections using the myofibrillar *adenosinetriphosphatase* (mATPase) method after acid (pH 4.3 and 4.6) and alkali (pH 10.5) preincubation according to Guth and Samaha (1970), with minor modifications (Muentener 1979). Subsequently, we classified the muscle fibres according to their MYH isoform into MYH-1 and MYH-2, measured the muscle fibres' diameter by the lesser fibre diameter-method (Dubowitz and Sewry 2007), and estimated fibre CSA from the lesser diameter assuming the fibres to be circular. Composition of MYH type 1 and type 2 fibres was determined from ATPase stains.

For the analysis of oxidative enzyme activity we incubated consecutive sections in media containing *cytochrome c oxidase*. For the determination of muscle capillarisation, we fixed consecutive cryocut sections with acetone for 10 min at 4 °C and exposed them to the monoclonal mouse anti-human CD31 endothelial cell antibody (DAKO, Carpinteria, Canada, 1:600 dilution), which acts as a marker for muscle capillarisation. We performed this staining procedure using the Vectastain® ABC Kit (Vector Laboratories, Burlingame, Canada), according to the manufacturer's instructions. For the control samples, we replaced the antibody with Tris-buffered saline (TBS) buffer. Overall capillary-to-fibre ratio was calculated by dividing the number of CD31-positive cells by the number of muscle fibres. Images were captured by an optical widefield microscope (Polyvar, Reichert-Jung, Vienna, Austria) with a connected digital camera (Leica DFC 420 C, Wetzlar, Germany)

under the same microscope objective (10×). For all histochemical and immunohistochemical analyses, we used the NIH Image J software (version 1.410, National Institutes of Health, Bethesda, MD, USA). Arbitrary *cytochrome c* enzyme activity levels were derived from the measured mean optical density pixel values of the muscle fibres normalised to the background pixel values on the same section.

Dual-energy X-ray absorptiometry (DXA). We used DXA (Lunar iDXA™, GE Healthcare, Madison, WI, USA) to determine the lean mass of both thighs. Scans were conducted in the standard mode (0.13-0.25 m) according to the manufacturer's specifications. We used the GE encore software version 11.40.004 to delineate the regions of interest (ROI, *i.e.* thigh) as follows: ROI upper boundary = line through the femoral neck parallel to the inguinal ligament; lower boundary = horizontal line between femur and tibia; lateral boundaries = outer leg cuts.

Oral glucose tolerance test. A standard glucose tolerance test (OGTT) was performed in the morning after an overnight fast. Participants ingested 75 g of glucose in 300 ml water. Venous blood samples were drawn two times just before and at 30, 60, 90, and 120 min after ingestion of glucose for determining plasma glucose, serum insulin, and C-petide.

Blood analysis. Venous blood samples were collected in EDTA, separated, and stored at −20°C before being assayed. Plasma glucose concentrations were determined by an automated *hexokinase* method (HK, Unit-Kit III, Roche, Basel, Switzerland) and serum insulin concentrations were determined by radioimmunoassay (Insulin ct-kit, Cisbio Bioassays, Bagnols-sur-Cèze Cedex, France). Haemoglobin A1c (HbA1c) was immunochemically measured with the DXC 2000® device (Bayer, Leverkusen, Germany). The high sensitivity c-reactive protein (hsCRP) was assessed with the Immulite 2500 kit (Siemens, Siemens Medical Solution Diagnostics, Los Angeles, USA), triglyceride, high-density lipoprotein (HDL), and cholesterol were analysed using commercial tests from Roche Diagnostics GmbH (Basel, Switzerland). Insulin sensitivity was estimated by the homeostasis model assessment (HOMA-IR) and calculated as: HOMA-IR = {[fasting insulin (μU·ml $^{-1}$)]·[fasting glucose (mmol·l^{-1})]}·22.5^{-1}.

Training. Training loads and number of completed repetitions (9-10 s of TUT per repetition, as described before) were continuously recorded. Training performance was retrospectively assessed by comparing loads and repetitions for the first set of VRO squat exercise between the first and last progressive training session. These measures were chosen because 1-repetition maximum testing is not applicable to VRO. During the third and last training session, we continuously recorded heart rate (S610i, Polar Electro, Kempele, Finland), and measured blood lactate concentration by drawing 20 µl of arterialised venous blood from an earlobe at rest, after the squat routine, after the heel rise routine, and 10-15 min after exercise. Blood samples were analysed enzymatically amperometrically with a BIOSEN C_line Sport® (EKF-diagnostic, Barleben, Germany). In addition, we assessed perceived exertion and perceived pain after the third squat and heel rise duty cycle using a visual analogue scale (VAS). The VAS scale consisted of a horizontal line. The word "none" was placed at the left end of the scale, and "very severe" was placed at the right end of the scale. The VAS was scored from 0 to 10, but the participants were unaware of the numbers.

Isokinetic dynamometry. We tested maximal knee extension power and fatigue resistance of the nondominant leg using a commercially available dynamometer (Con-Trex MJ, CMV, Dubendorf, Switzerland). The inclination of the backrest was set to an angle of 85°. The dominant leg was fixed at a knee angle of 90° and body posture was stabilised using straps and handles. The individual maximal ROM was determined during the pretests and also used for posttests. Before the tests, the participants performed 20 warm-up movements, alternating between knee extensions and flexions of varying submaximal effort and angular velocity. Warm-up was followed by two maximal knee extensions (ω = 3.14 rad·s^{-1}), separated by 1 min, to assess maximal power. Subsequently, a fatigue test consisting of 50 alternate maximal voluntary knee extensions and knee flexions (ω = 3.14 rad·s^{-1}) was performed to assess end-test leg extension torque (mean value over the last five repetitions) and to calculate fatigue percentage [(mean torque value of the first five maximal repetitions – end-test torque)/(mean torque value of the first 5 maximal repetitions) ·100].

Jumping mechanography. Three vertical countermovement jumps with freely moving arms (separated by 30 s of rest) were performed on a strain gauge ground reaction force platform (Leonardo®, Novotec, Pforzheim, Germany) linked to a

desktop computer using an integrated analog digital board and software system (Leonardo® GRFP Mechanography version 4.2, Novotec, Pforzheim, Germany). The participants were instructed to remove their shoes, stand with feet shoulder width apart and arms hanging loosely at their sides. They were further instructed to jump as high as possible while keeping their head still during the flight of the jump, and to stand still again with arms hanging loosely after landing. The maximum jump height was calculated as the highest displacement of the center of mass, and the relative maximal power was calculated from the product of force and velocity. From the three valid attempts, the jump with the highest jump height was used for further analyses.

Graded cycling exercise tests. We used Innocor™ (Innovision, Odense, Denmark) to determine maximal cardiac output by inert gas rebreathing, oxygen consumption and ventilation by breath-by-breath ergospirometry, as well as arterial oxygen saturation and heart rate during a GXT as previously described (Fontana et al. 2009). Participants rested in the seated position on the cycle ergometer for 2 min, and afterwards started cycling at 50 W with a self-selected pedal rate (≥ 70 revolutions·min^{-1}). This pedal rate was consistently (± 5 revolutions·min^{-1}) applied throughout the test. Power was increased by 25 W every 2 min until volitional exhaustion, *i.e.* the point in time at which the participants stopped cycling or were no longer able to maintain pedal rate within the required limits. Maximal cardiac output was determined immediately before exhaustion, and arterio-venous oxygen difference was calculated according to the Fick principle. Ventilatory threshold was determined as the power corresponding with a disproportionate increase in minute ventilation. In addition, we assessed rate of perceived exertion using a modified Borg scale from 0 to 10 at rest, at the end of each stage, and at exhaustion.

Constant-load cycling exercise tests. We used a constant-load cycling exercise test (CLT) to assess time to exhaustion at submaximal power as an indicator of endurance capacity. Power of the initial warm-up stage was 40% maximal power of the first GXT. After 1 min, we increased power to 60% peak power (for 2 min) and then to 85% maximal power. Power at 85% maximal power was sustained until volitional exhaustion, *i.e.* the point in time at which the participants stopped pedalling or were no longer able to maintain pedal rate within the required limits.

Statistical analyses. We report all group data as mean (SD). A paired sample *t*-test was used to compare intra-group differences. To assess between-group effects we analysed the differences between pre- and posttraining values using student's *t*-test for 2 independent samples. Equality of variances was checked using Levene's test. We used SPSS 16.0 statistical software (SPSS, Chicago, USA) for all statistical analyses, and statistical significance was set at $P < 0.05$.

Results

Training

Maximal heart rate achieved during VRO was 94.1 and 91.4 % of maximal heart rate during GXT for pre- and posttraining measurements, respectively (Fig. 4.2A). The corresponding values for maximal blood lactate concentration were 77.3 and 75.4% (Fig. 4.2B). Perceived exertion and pain were high and did not change over time, indicating that participants exercised with maximum voluntary effort (Fig. 4.2C). Perceived exertion during VRO was similar to maximal exercise during GXT to exhaustion, in which perceived exertion was rated 9.7 (0.9) and 9.5 (1.2) on a modified Borg scale for pre- and postmeasurements, respectively. During the period of time between the first (occurring in week 1) and last (occurring in week 6) progressive training session, VRO participants increased the training load by 84.5%, while the number of repetitions (and as such TUT) until volitional failure was the same at both points in time (Fig. 4.2D).

Muscle (fibre) size, metabolism, and capillarisation

VRO resulted in a 16.7 and 13.8% increase in *vastus lateralis* muscle fibre CSA for MYH type 1 and 2 fibres, respectively (Fig. 4.3A, B). However, a testing time by group interaction was observed only for MYH-1 fibre hypertrophy ($P = 0.019$), demonstrating that VRO increased MYH-1 fibre CSA relative to CON. Concordantly,

thigh lean mass increased by 4% in the VRO group [VRO: 10.6 vs. 11.0 kg (P = 0.001); CON: 10.7 vs. 10.7 (P = 0.972); testing time by group interaction: P = 0.008] (Fig. 4.3C). Notably, MYH-1 fibre proportion and overall capillary-to-fibre ratio were increased by 14.0% each (Fig. 4.3D). Furthermore, the increase in *cytochrome c oxidase* activity reflecting the shift in the MYH-1 and MYH-2 fibres' metabolic phenotype towards the oxidative phenotype was only observed in VRO (Fig. 4.3E, F).

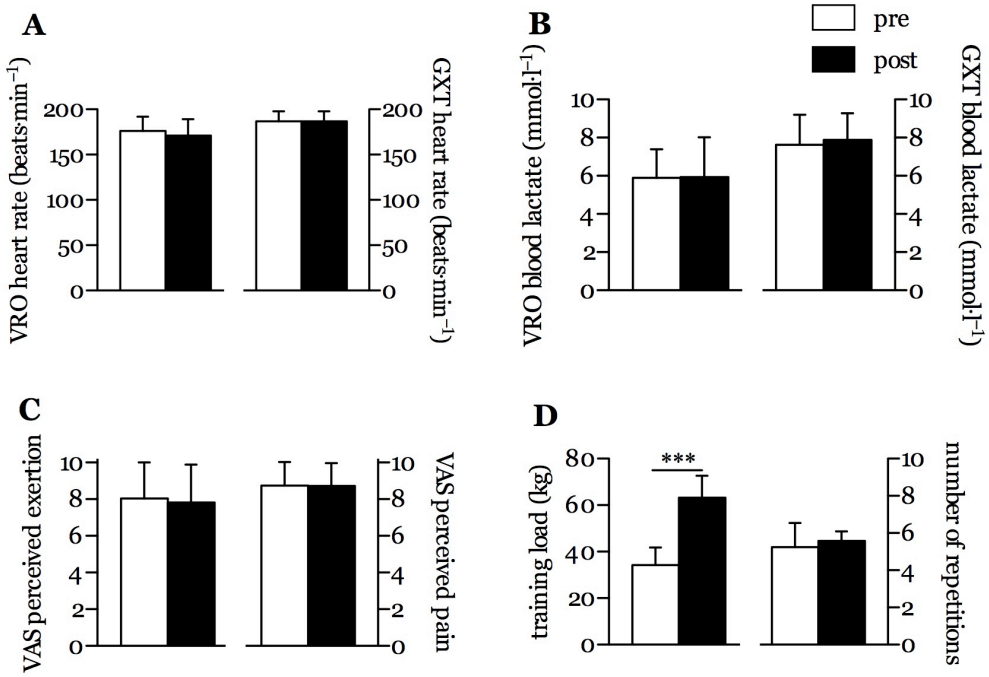

Figure 4.2: Peak heart rate (A) and blood lactate concentration (B) during VRO compared to the maximal values obtained during the graded cycling exercise test (GXT) at the beginning and end of the study. Perceived exertion and pain during the 3rd VRO squat set at the beginning and end of the study (C). Training load and number of repetitions (9-10 s per repetition) during the first squat set of the first and last training session (D). Bars and error bars represent mean values and standard deviations, respectively, for the 12 women undergoing VRO. VRO, Galileo® vibration + resistance exercise + vascular occlusion. ***, significantly different first vs. last training session at P < 0.001.

Submaximal and maximal power, time to exhaustion, and end-test torque

Maximal power during GXT was increased by 8.7% in VRO relative to CON (Fig. 4.4A). VRO also increased time to exhaustion during CLT and ventilatory threshold (GXT) by 57.0 and 11.7%, respectively (Fig. 4.4B,C), relative to CON. Additionally, VRO improved concentric isokinetic leg extension fatigue resistance by 15% (Fig. 4.4D), while neither maximal concentric isokinetic knee extension power nor maximal jumping power per kg body mass was increased after VRO (Fig. 4.4E, F). Body mass remained unchanged for both groups [VRO: 60.9 (8.1) vs. 61.1 (8.0), P = 0.680; CON: 60.7 (5.4) vs. 60.7 (5.6), P = 1.000].

Cardiac output, oxygen consumption, and ventilation

VRO increased maximal cardiac output (albeit without significant testing time by group interaction) (Tab. 4.1), while no change in maximal oxygen consumption, calculated arterio-venous oxygen difference, or arterial oxygen saturation was observed for either group. Higher maximal power in VRO had no effect on ventilation, while a lower maximal power correlated with a reduction in ventilation in CON (Tab. 4.1).

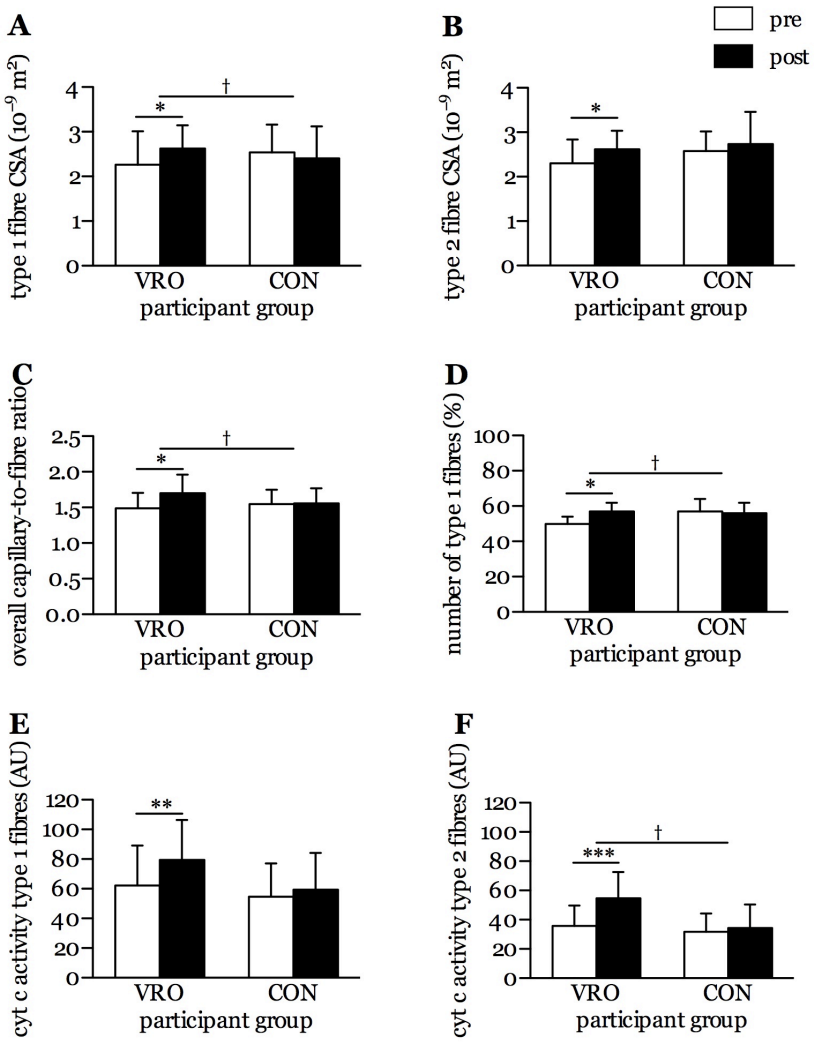

Figure 4.3: Muscle fibre cross-sectional area (CSA) for myosin heavy chain type 1 (A) and 2 (B) fibres, overall capillary-to-fibre ratio (C), myosin heavy chain type 1 fibre percentage (D), and *cytochrome c* (cyt c) *oxidase* activity in optical arbitrary units (AU) for type 1 (E) and 2 (F) fibres pre- and posttraining. Bars and error bars represent mean values and standard deviations, respectively, for: (A) and (B), eight women per group; (C), seven women per group; (D), six and five women in VRO and CON, respectively; (E) and (F), eight and seven women in VRO and CON, respectively. VRO, Galileo® vibration + resistance exercise + vascular occlusion; CON, control group. *,**, ***, significantly different within group pre vs. post, *P < 0.05, **P < 0.01, ***P < 0.001; †, pre vs. post differences significantly different between groups at P < 0.05.

Table 4.1: Maximal values for cardiac output, oxygen consumption, and ventilation during the graded exercise test. Values are means (SD) for 12 women (VRO) and nine women (CON). VRO, Galileo® vibration + resistance exercise + vascular occlusion; CON, control group. *P < 0.05, **P < 0.01; †, pre vs. post differences significantly different between groups at P < 0.05.

	VRO		CON		
	Pre	Post	Pre	Post	VRO vs. CON
Cardiac output ($l \cdot min^{-1}$)	12.3 (2.2)	13.4 (1.7)	11.9 (1.7)	12.4 (2.3)	
Arterio-venous oxygen difference ($ml\ O_2 \cdot 100\ ml^{-1}$)	18.2 (2.4)	17.2 (2.1)	18.5 (2.6)	17.1 (2.5)	
Arterial oxygen saturation (%)	96.3 (1.6)	95.5 (2.5)	96.7 (1.9)	96.7 (1.9)	
Oxygen consumption ($l \cdot min^{-1}$)	2.21 (0.25)	2.27 (0.14)	2.21 (0.48)	2.10 (0.34	
Ventilation ($l \cdot min^{-1}$)	79 (15)	83 (13)	78 (16)	71 (17)**	†

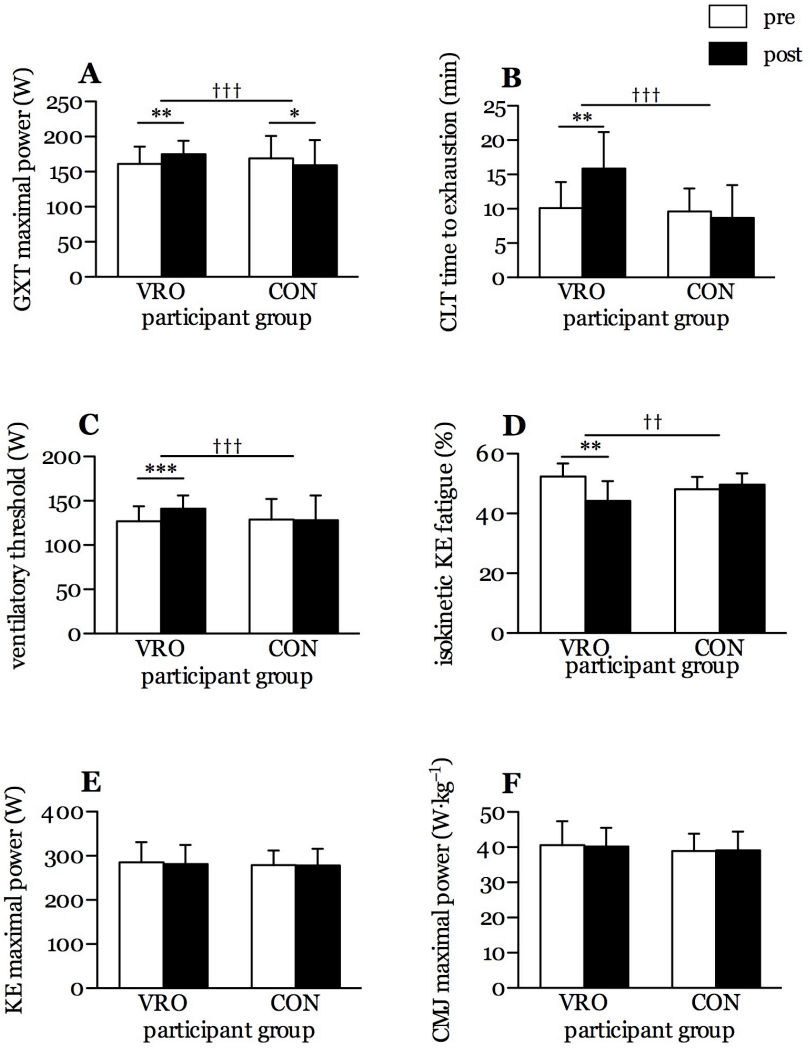

Figure 4.4: Maximal power during the graded cycling exercise test (GXT, A), time to exhaustion during a constant-load cycling exercise test (CLT, B), ventilatory threshold calculated based on gas exchange during GXT (C), knee extension (KE) fatigue following 50 repeated maximal concentric isokinetic (ω = 3.14 rad·s^{-1}) knee extensions (D), maximal concentric isokinetic knee extension power (E), and maximal countermovement jump (CMJ) power per kg body mass (F) pre- and posttraining. Bars and error bars represent mean values and standard deviations, respectively, for: (A), (B), (C), and (F), 12 and nine women in VRO and CON, respectively; (D) and (E), 10 and seven women in VRO and CON, respectively. VRO, Galileo® vibration + resistance exercise + vascular occlusion;. VRO, synthetic exercise group; CON, control group. *, **, ***, significantly different pre vs. post within group, *P < 0.05, **P < 0.01, ***P < 0.001; ††, †††, pre vs. post differences significantly different between groups, ††P < 0.01, †††P < 0.001.

Body composition

VRO resulted in a 2.0 and 4.5% increase in total body and leg lean masses, respectively (Tab. 2.2). However, a testing time by group interaction was observed only for total leg lean mass (P = 0.001), indicating that VRO increased leg lean mass relative to CON. Concordantly, total body fat mass and relative body fat decreased solely after VRO, with a group interaction in relative body fat (P = 0.035) (Tab. 2.2). These changes in body composition led to an unaffected total body mass (Tab. 2.2).

Table 2.2: Body composition of study participants

	VRO		CON		
	Pre	Post	Pre	Post	VRO vs. CON
Body mass (kg)	60.9 (8.1)	61.1 (8.0)	60.7 (5.4)	60.7 (5.6)	
Body lean mass (kg)	40.2 (4.6)	41.0 (4.7)**	40.6 (4.0)	40.6 (4.2)	
Abdominal lean mass (kg)	2.4 (0.3)	2.5 (0.3)	2.5 (0.3)	2.5 (0.3)	
Gynoid lean mass (kg)	5.5 (0.6)	5.6 (0.5)*	5.5 (0.6)	5.5 (0.6)	
Leg lean mass (kg)	14.0 (1.8)	14.6 (1.8)***	14.1 (1.5)	14.1 (1.4)	††
Thigh lean mass (kg)	12.3 (1.6)	12.8 (1.5)***	12.4 (1.3)	12.4 (1.3)	††
Lower leg lean mass (kg)	1.7 (0.3)	1.8 (0.3)***	1.7 (0.2)	1.7 (0.2)	†††
Body fat mass (kg)	18.8 (4.4)	18.2 (4.2)*	18.5 (4.0)	18.4 (4.4)	
Body fat (%)	31.6 (4.2)	30.5 (3.9) **	31.2 (5.2)	31.0 (5.9)	†
Abdominal fat mass (kg)	1.3 (0.5)	1.2 (0.4)	1.3 (0.6)	1.3 (0.6)	
Gynoid fat mass (kg)	4.7 (1.0)	4.6 (0.9)	4.6 (0.7)	4.6 (0.9)	
Leg fat mass (kg)	8.1 (1.8)	7.9 (1.8)	7.6 (1.1)	7.7 (0.8)	

Values are means (SD) for 12 women (VRO) and nine women (CON). VRO, Galileo® vibration + resistance exercise + vascular occlusion; CON, control group. *,**, ***, significantly different within group pre vs. post, *P < 0.05, **P < 0.01, ***P < 0.001; †,††,†††, pre vs. post differences significantly different between groups at †P < 0.05, ††P < 0.01, †††P < 0.001.

Cardiometabolic risk factors

Cardiometabolic risk factors such as waist-to-hip ratio, blood pressure, blood lipid levels, glycaemic status, calculated insulin resistance, and blood glucose concentration in the fasting state as well as after the ingestion of glucose were unaffected after both training regimens, VRO and RES, respectively (Tab. 2.3).

Table 2.3: Cardiometabolic risk factors of study participants

	EXE		CON	
	Pre	Post	Pre	Post
Waist-to-hip ratio	0.70 (0.04)	0.71 (0.06)	0.74 (0.04)	0.74 (0.04)
Body mass index ($kg \cdot m^{-2}$)	21.6 (2.6)	21.7 (2.7)	21.8 (1.6)	21.8 (1.7)
Systolic blood pressure (mmHg)	119 (11)	120 (11)	122 (7)	118 (6)
Diastolic blood pressure (mmHg)	77 (8)	77 (10)	76 (7)	74 (4)
Triglycerides ($mmol \cdot l^{-1}$)	0.88 (0.33)	0.95 (0.43)	0.74 (0.34)	0.74 (0.28)
HDL-C ($mmol \cdot l^{-1}$)	1.66 (0.27)	1.67 (0.22)	1.70 (0.36)	1.57 (0.40)
Total-C ($mmol \cdot l^{-1}$)	4.49 (1.05)	4.43 (0.89)	4.67 (0.69)	4.37 (0.77)*
Total-C·HDL-C^{-1}	2.74 (0.72)	2.64 (0.55)	2.83 (0.49)	2.91 (0.61)
hsCRP ($mg \cdot l^{-1}$)	1.2 (0.7)	0.8 (0.7)	2.7 (3.8)	2.6 (1.3)
HbA1c (%)	5.3 (0.2)	5.2 (0.2)	5.2 (0.1)	5.2 (0.1)
HOMA-IR	1.33 (0.33)	1.35 (0.44)	1.38 (0.47)	1.30 (0.44)
Fasting plasma glucose ($mmol \cdot l^{-1}$)	4.49 (0.28)	4.57 (0.27)	4.49 (0.24)	4.32 (0.28)
OGTT - Plasma glucose AUC ($mmol \cdot min \cdot l^{-1}$)	686 (138)	711 (125)	674 (96)	640 (100)
OGTT - Plasma insulin AUC ($pmol \cdot min \cdot l^{-1} \cdot 10^3$)	51 (13)	50 (14)	53 (14)	49 (14)
OGTT - Plasma C-peptide AUC ($pmol \cdot min \cdot l^{-1} \cdot 10^3$)	265 (57)	280 (60)	274 (56)	255 (62)

Values are means (SD). VRO, Galileo® vibration + resistance exercise + vascular occlusion; CON, control group; HDL-C, high-density lipoprotein cholesterol; total-C, total cholesterol; hsCRP, high-sensitive c-reactive protein; HbA1c, glycosylated haemoglobin A1c; HOMA-IR, homeostasis model assessment, calculated insulin resistance; OGTT, oral glucose tolerance test; AUC, area under the curve. Significantly different within group pre vs. post, $^*P < 0.05$.

Discussion

This study showed that 5 weeks of VRO significantly increased the maximal cycling power, time to exhaustion at submaximal cycling power, and end-test torque after 50 maximal concentric isokinetic (ω = 3.14 rad·s^{-1}) leg extensions in young women. However, VRO had no effect on their maximal jumping and concentric isokinetic leg extension power. Increased endurance capacity was commensurate with increased *vastus lateralis* muscle MYH-1 fibre proportion and hypertrophy, oxidative enzymes, and overall capillarisation, while maximal oxygen consumption and cardiac output remained unchanged. Despite the increased total body and leg lean masses after VRO, no changes in cardiometabolic risk factors were observed. Our results demonstrate that VRO is an effective training modality to rapidly increase submaximal power capacity in young women. Furthermore, given that the *vastus lateralis* muscle adaptations were achieved with a minimal of 3 × 3 min of thigh muscle activity per week, time efficiency of VRO was high.

VRO is a training modality which required maximum effort from participants. This was evident from the high ratings of perceived exertion and pain, the high peak blood lactate concentration, and the high peak heart rate. In return, training time commitment for VRO was substantially lower (by as much as 90%) relative to classical endurance exercise. The increase in VRO training load for squats was substantial. It increased by 85% in 5 weeks, which corresponds to a 15 percentage points higher value than that previously reported for the increase (approximately 70% within 5 weeks of training) in squat 1-RM during the early phase of heavy-resistance training in young women (Staron et al. 1994, calculated from Fig. 4.2C). The increase in training load during VRO was linear, with 70% of the overall gain occurring within the first 20 (of 35) days of training (data not shown). To our knowledge, there are no reports about the time course of squat training load adaptation in young women during the first 5 weeks of training. However, in young men undergoing flywheel resistance training, investigators have found that approximately 65% of the increase occurs within the first 10 (of 35) days of training

(Seynnes et al. 2007). Therefore, the increase in training load during the early phase (first 10 days) of VRO apparently was less steep relative to classical resistance exercise, but it was steeper for the remainder of the training duration (35 days). The observed time course thus suggests that neural adaptations and gains in muscle CSA following VRO might be different in nature and/or time course of manifestation relative to classical resistance exercise.

The increase in training load following VRO correlated with increases in muscle fibre CSA. It is widely accepted that resistive exercise can increase the CSA of both, type 1 and 2 fibres. For example, it has been shown that CSA of type 1 and 2A (but not 2X) fibres in the *vastus lateralis* muscle of young women increases by 20 and 19%, respectively, after 9 weeks of high-resistance exercise, using a protocol which requires an individual to perform 50 unilateral leg extension repetitions at near-maximal effort per session for 3 sessions per week (*i.e.* 150 near-maximal repetitions per week, Martel et al. 2006). In comparison, VRO increased CSA of type 1 and 2 fibres to a similar extent (17 and 14% for type 1 and 2 fibres, respectively) in approximately half the time, and with a training volume that was approximately 2/3 lower (18 repetitions per session for 3 session per week, *i.e.* 54 repetitions per week). However, only MYH-1 fibre hypertrophy was significant between groups over time (Fig. 4.3A, B), indicating that 5 weeks of VRO preferentially promoted type 1 fibre hypertrophy. There are also reports of greater increases in CSA for all fibre types, as determined in *vastus lateralis* muscle biopsy samples from young women after 6 weeks of high-resistance exercise (Staron et al. 1991). However, in that study 3 different exercise modes comprising both open- (leg extension) and closed-chain (squat and leg press) actions were employed to induce hypertrophy. This might facilitate the detection of a higher number of size-enlarged fibres in the sampled muscle tissues because of the increased motor unit recruitment coverage (and consequently the number of stimulated *vastus lateralis* muscle fibres) during training relative to squat exercise only. Notably, VRO mediated a fast adaptation in muscle fibre CSA, considering that even in the case of high-resistance exercise with different exercise modes (open- and closed-chain) and larger training volume, increases in CSA of *vastus lateralis* muscle fibres in young women usually require at least 6-8 weeks of training (Staron et al. 1994, Staron et al. 1991).

DXA thigh lean mass increased by approximately 4% in 5 weeks (Tab. 2.2), lending further credence to our finding that VRO induced muscle fibre hypertrophy. Other

investigators have recently found that 12 weeks (5 training sessions per week) of heavy, whole-body resistance exercise, consisting of a rotating multiset and multimode split routine targeting all major muscle groups, leads to an approximate 5% increase in total body lean mass in young women supplementing their diets with 0.5 l fat-free milk immediately post and 1 h post exercise (2 times 0.5 l) (Josse et al. 2010, calculated from Fig. 2). In contrast, women consuming isoenergetic carbohydrate instead of protein increased total body lean mass by only 2.5% (Josse et al. 2010, calculated from Fig. 2). Similarly, in elderly men and women a 2.9% increase in DXA-derived thigh muscle mass was found following resistance exercise requiring an individual to perform 135 leg extensions 3 times per week for 10 weeks (*i.e.* 405 repetitions per week, 4050 repetitions in total, Delmonico et al. 2008). Hence, assuming that segmental training-induced increases in DXA muscle mass are proportional to total lean mass increases after whole-body exercise, an increase of 2.5-3.0% in DXA thigh muscle mass can be expected after 10-12 weeks of high-resistance exercise when the participants' diets are not supplemented with protein in the postexercise period. Our finding that the participants undergoing VRO increased thigh lean mass by 4% in half the time without supplementing protein and furthermore using a training volume which can be up to 15 times smaller (i.e. 270 vs. 4050 repetitions) indicates that VRO may lead to an increased net muscle protein balance relative to classical resistance exercise. However, changes in DXA lean mass should be interpreted with caution, because DXA technology cannot discriminate between the various components (water, protein, glycogen, and minerals) within the lean soft tissue.

Increases in training load and muscle fibre CSA are generalised findings for training modalities involving high-load (> 70% 1-RM) resistance or low-load (20–50% 1-RM) resistance combined with blood flow restriction. As a novel and specific feature, VRO concurrently increased MYH-1 fibre proportion, overall capillary-to-fibre ratio, and oxidative enzyme activity. The finding that VRO increased MYH-1 fibre proportion is of special interest, since the only conversion of pure fibre types following endurance and/or resistance exercise in humans that is well documented is conversion of type 2X to type 2A, and thus it is not believed that type 2 fibres can be converted to type 1 fibres with classical training (Spangenburg and Booth 2003).

Whereas capillarisation can increase after weeks or months of moderate to vigorous endurance training in humans (Andersen and Henriksson 1977), high-resistance

exercise usually decreases capillary density, presumably as a result of fibre hypertrophy in the absence of angiogenesis (Egginton 2009). Specifically, in young women both the overall capillary-to-fibre ratio and the capillary density are not influenced even after 18 weeks of resistance exercise (Wang et al. 1993). Similar to the adaptation in capillarisation, increases in mitochondrial enzyme levels and respiratory capacity are common adaptations to endurance exercise (Holloszy and Coyle 1984) but generally remain unaffected in response to resistance-type exercise (Tesch 1988).

Taken together, VRO concurrently induced muscular adaptations that typically are mediated by either high-resistance (muscle hypertrophy) or endurance exercise (capillarisation, oxidative enzymes). In addition, like 1-legged cycling exercise (45 min per session, four sessions per week for 4 weeks) with 50 mmHg (6.67 kPa) external pressure over the working leg (Sundberg 1994) VRO increased the proportion of MYH-1 fibres. These cellular/organic changes were paralleled by an increase in critical torque, as shown by the increased end-test torque following 50 consecutive maximal isokinetic (ω = 3.14 rad·s^{-1}) knee extensions. End-test torque has been shown to correlate with the estimated critical torque (Burnley 2009), which in turn has been suggested to be analogous to the critical power for cycling exercise (Burnley 2009, Jones et al. 2010). Our finding that end-test torque was increased by 15% after VRO is consistent with those of Eiken et al. (1991) and Takarada et al. (2002) who found that isokinetic (ω = 3.14 rad·s^{-1}) end-test torque is increased by 13 and 7.6 % after a period of ischaemic 1-legged cycling exercise and low-resistance exercise with vascular occlusion, respectively. Consistent with the notion that dynamometry-derived critical torque is analogous to critical power for cycling exercise, both the increase in ventilatory threshold and the markedly (+ 57%) increased time to exhaustion during a CLT could be explained by an increase in critical power in this study. Notably, we found that endurance capacity could be increased without concurrently increasing maximal oxygen consumption, supporting the conclusion of other authors that the percentage change in submaximal performance is not associated with the percentage change in maximal oxygen consumption (Vollaard et al. 2009). In contrast to the marked increases in the capability of participant to generate and sustain submaximal power, maximal power remained unchanged, as shown by the similar values for maximum voluntary isokinetic (ω = 3.14 rad·s^{-1}) leg extension power and jumping power normalised to body mass (body mass remained unchanged). This finding contrasts the current

notion that vibration exercise alone or in combination with resistance exercise increases knee extension and jumping power (Rittweger 2010), and points towards a distinct role for vascular occlusion. The fact that maximal concentric isokinetic (ω = 3.14 rad·s^{-1}) leg extension power remained unchanged after VRO indicates that muscle force also remained constant (power = force · velocity). Unchanged muscle force can be explained by a concurrent increase in MYH-1 fibre CSA and proportion after VRO. In fact, the increase in maximal concentric isokinetic force due to an increase in the number of parallel sarcomeres within MYH-1 fibres might have been blunted by the concurrent increase in the share of the muscle CSA consisting of MYH-1 fibres, because MYH-1 fibres have reduced isometric maximal tension (Linari et al. 2004) relative to MYH-2 fibres. Moreover, MYH-1 fibres display lower maximal shortening velocity and power than MYH-2 fibres (Linari et al. 2004). Thus, despite the higher MYH-1 fibre power due to hypertrophy, mixed muscle maximal shortening velocity might be reduced, possibly explaining the unchanged maximal jumping power after VRO. Similarly, Eiken et al. (1991) found that the increase in MYH-1 fibre proportion after ischaemic training (notably without concurrent fibre hypertrophy) was paralleled by a decreased maximal isokinetic (ω = 3.14 rad·s^{-1}) leg extension torque. They concluded that blood flow-restricted training, in contrast to non-restricted-flow training, decreases maximum torque because of an increase in the share of the muscle CSA consisting of MYH-1 fibres. In our study, fibre hypertrophy might have prevented the drop in maximal power otherwise caused in the case of a sole increase in MYH-1 fibre proportion.

Despite the increase in total body and leg lean masses by 2.0 and 4.5% and the decrease in total fat mass by 3.2% after VRO, no changes in various cardiometabolic risk factors were observed (Tab. 2.2 and 2.3). All study participants were healthy and asymptomatic without a cardiometabolic risk factor clustering (Wildman et al. 2008). The mechanism how physical activity influences cardiometabolic risk factors is only partially understood. It has been suggested that glucose uptake after endurance and resistance exercise training can occur with and without changes in total lean mass and body composition (Poehlman et al. 2000). Based on the fact, that VRO was consisted of a very short-time training invervention, our data indicate that not solely the skeletal muscle mass makes a major impact on cardiometabolic risk factors but that the time under which skeletal muscles are under tension is more important.

In summary, 5 weeks of VRO concurrently increased GXT power, CLT time to exhaustion, ventilatory threshold and end-test torque. These increases were related to increases in MYH-1 fibre hypertrophy and proportion, muscle capillarisation, and oxidative enzymes, but not maximal oxygen consumption and cardiac output. Conversely, VRO had no effect on maximal isokinetic leg extension and jumping power, probably due to the competing effects of MYH-1 fibre hypertrophy and increased MYH-1 fibre proportion on maximal mixed muscle tension and velocity. We conclude that VRO is an effective and time efficient (*vastus lateralis* muscle adaptations were achieved with a minimal of 3 × 3 min of thigh muscle activity per week) training modality to quickly increase the capacity to generate and sustain submaximal power in young women.

Chapter 5

Modified resistance exercise increases PGC-1α and VEGF mRNA abundances

Flurin Item[1,2], Antonio Nocito[3], Sandra Thoeny[1], Thomas Baechler[3], Urs Boutellier[1,2], Roland H. Wenger [2], and Marco Toigo[1,2]

[1] Exercise Physiology, Institute of Human Movement Sciences and Sport, ETH Zurich, Zurich, Switzerland
[2] Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland
[3] Department of Visceral and Transplantation Surgery, University Hospital Zurich, Zurich, Switzerland

Introduction

Endurance exercise is a powerful inducer of mitochondrial biogenesis and angiogenesis in human skeletal muscle (Coffey and Hawley 2007). Mitochondrial biogenesis involves the coordinated expression of the mitochondrial genome and the nuclear genes that encode mitochondrial proteins (Hood 2001), while angiogenesis requires the coordinated action of numerous signals involved in vessel formation and maturation (Carmeliet 2000). One transcriptional regulator that can orchestrate both signals required for mitochondrial biogenesis and angiogenesis is peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (Arany et al. 2008, Geng et al. 2010, Puigserver and Spiegelman 2003, St-Pierre et al. 2003, Wu et al. 1999). PGC-1α docks on and coactivates the nuclear respiratory factors 1 and 2α (NRF1 and NRF2α) (Wu et al. 1999). These transcription factors regulate the expression of nuclear genes encoding mitochondrial proteins (Scarpulla 2002) and induce the expression of mitochondrial transcription factor A (Tfam) (Wu et al. 1999). PGC-1α also activates the transcription factor estrogen-related receptor α (ERRα), which elicits a robust induction of vascular endothelial growth factor (VEGF) in cultured muscle cells and mouse skeletal muscle *in vivo* following hypoxia and limb ischaemia, respectively (Arany et al. 2008). Arany et al. (2008) concluded that PGC-1α has a critical function in the angiogenic response to ischaemia and they speculated that the PGC-1α/ERRα pathway also mediates exercise-induced neovascularisation (Arany et al. 2008). Indeed, it has recently been shown that in rodent skeletal muscle, exercise-induced PGC-1α regulates the vascular endothelial growth factor (VEGF) (Chinsomboon et al. 2009, Geng et al. 2010). Notably, the induction of VEGF by PGC-1α has been shown to occur independent of hypoxia-inducible factor-1α (HIF-1α) (Arany et al. 2008), which is a well-known hypoxia-induced transcription factor regulating VEGF expression (Wenger 2000). Increased PGC-1α mRNA expression after a single bout of endurance exercise is related to AMP-activated protein kinase (AMPK) activation (Jaeger et al. 2007), p38 mitogen-activated protein kinase activation (Akimoto et al. 2005, Puigserver et al. 2001), calcium signalling (Handschin et al. 2003), and the level of reactive oxygen species

(ROS) (St-Pierre et al. 2006), indicating that both metabolic and oxidative stress are responsible for the induction of PGC-1α.

In Chapter 4, we reported that 5 weeks of whole-body vibration training with superimposed heavy resistance exercise and sustained vascular occlusion (VRO) increases capillary-to-fibre ratio, skeletal muscle oxidative enzyme activity, myosin heavy chain type 1 (MYH-1) fibre proportion, and endurance capacity. These findings were intriguing, since increases in oxidative muscle phenotype and endurance capacity are typically induced by endurance exercise but not heavy resistance exercise (Coffey and Hawley 2007, Holloszy and Coyle 1984). Contrary to classical heavy resistance exercise, VRO comprises sustained ischaemia and vibration. These two stimuli have been shown to activate VEGF expression and promote capillarisation in various settings (Gustafsson et al. 1999, 2002, Richardson et al. 1999, Sundberg 1999). We thus hypothesised that a single bout of VRO activates several genes associated with mitochondrial biogenesis and angiogenesis. To this end, we analysed the acute effects of VRO on gene expression in comparison to traditional heavy resistance exercise.

Design and Methods

Participants

Eight men of age 23.1 (2.8) years participated in this study. They were all healthy, asymptomatic, non-smoking, and recreationally active. After completing a routine health questionnaire the participants were informed about the applied procedures and the associated risks. Informed written consent was obtained from all participants. The experimental protocol was approved by the ethics committee of the canton of Zurich, and the study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki for human experimentation.

Experimental protocol

The study consisted of a crossover design. All subjects performed two different exercise protocols, which were separated by 2 weeks in a randomised order. Before the first exercise session, we performed baseline measurements, a familiarisation session, and a baseline biopsy, each session separated by 1 week. During the baseline measurements, we assessed body composition and squat 1RM. Participants could familiarise themselves with the experimental model and the associated procedures on a separate occasion. Participants were asked to refrain from vigorous physical activity for 24 h prior to all sessions accomplished in this study. The evening (12 h) before the baseline biopsy and exercise sessions, participants were fed a standardised meal consisting 2.96 g carbohydrate·kg^{-1} body mass, 0.5 g protein·kg^{-1} body mass, and 0.32 g fat·kg^{-1} body mass.

Exercise protocols

VRO. VRO has been described in Chapter 4 in detail. It was comprised of loaded (Multipower®, Technogym, Gambettola, Italy) squats standing on a Galileo® side-alternating vibration plate (Novotec, Pforzheim, Germany) oscillating at 30 Hz, and superimposed vascular occlusion. Load magnitude (approximately 70% of the individual 1RM) was adjusted from prior knowledge (familiarisation) in order to induce volitional failure within 60-100 s of exercise. Volitional exhaustion (task failure) was defined as the point in time where the participants failed to extend their legs. Vascular occlusion was induced by inflating tourniquet cuffs (VBM, Sulz a.N., Germany) affixed to the inguinal fold region to the thigh to approximately 200 mmHg (26.7 kPa). The suprasystolic pressure employed here was the highest pressure that was tolerated by the participants in this setting. The exercise regimen consisted of two sets, each set with a duty cycle of 4 min and 1 min off. During the duty cycle, participants performed loaded squats with superimposed whole-body vibration and vascular occlusion until volitional failure. Subsequently, tourniquet cuffs remained inflated until the 4 min of the duty cycle were complete. The cuffs were then deflated to a pressure of 100 mmHg (13.3 kPa), and participants rested

for 1 min before cuffs were reinflated for the second set. The load magnitude was reduced by approximately 10% points for the second set in order to keep exercise time constant.

RES. During RES, squats were performed without superimposed vibration and vascular occlusion, but while standing on the vibration plate (off). After volitional exhaustion, participants rested for 4 min standing still until the second set started.

Skeletal muscle biopsies

We obtained percutaneous biopsies from the middle region of the nondominant *vastus lateralis* muscle at baseline and 3 h post exercise using a 6-mm Bergstroem needle (Dixons Surgical Instruments, Essex, UK) with suction applied. Biopsy samples were immediately frozen in liquid nitrogen and stored at −80°C until mRNA were analysed. Part of the baseline biopsy were additionally mounted in an embedding medium (Tissue-Tek®, Sakura, Zoeterwoude, The Netherlands) and snap frozen in isopentane cooled to −160°C with liquid nitrogen for subsequent immunohistochemical analysis.

RNA extraction and mRNA analyses

Total RNA from *vastus lateralis* muscle samples was extracted by using the RNeasy fibrous tissue minikit (Qiagen, Basel, Switzerland). First-strand complementary DNA synthesis was performed with 200 μg total RNA by using reverse transcriptase, and mRNA abundance was determined by real-time quantitative PCR by using a SybrGreen® qPCR reagent kit (Sigma, Buchs, Switzerland) in combination with the MX3000P light cycler (Stratagene, Amsterdam, The Netherlands). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. Equal input levels were verified by determining ribosomal protein L28 mRNA abundance, and data were expressed as ratios relative to L28 mRNA expression. Sequences of the primers are given in Table 5.1.

Table 5.1: PCR primer sequences for the measured gene transcripts

Gene	Accession No.	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
CS	BT007414	aggaagactgatccgcgata	catggacttgggcctttcta
ERRα	NM004451	tgccaattcagactctgtgc	cctcgagcatctccaagaac
HIF-1α	HSU22431	tcatccaagaagccctaacg	ttgtcttttgctccattcca
HKII	NM000189	tggctttgaagaccctcact	caaattctgtgcggaagtca
L28	NM000991	gcaattccttccgctacaac	tgttcttgcggatcatgtgt
LDHA	NM001135239	tgtgcctgtatggagtggaa	cccaaaatgcaaggaacact
MnSOD	M36693	ccctggaacctcacatcaac	agtcacgtttgatggcttcc
NRF1	NM001040110	atgcagcagggagctacagt	atgctcacagggatctggac
PFKm	J05533	gtccctggctcagacttcag	ccttaacaccaagccccttt
PGC-1α	AF159714	caagccaaaccaacaactttatctct	cacacttaaggtgcgttcaatagtc
PHD3	NM022073	atcgacaggctggtcctcta	cttggcatcccaattcttgt
Tfam	NM003201	ggaaaaccaaaaagacctcgttcagctt	ttttcctgcggtgaatcaccctt
VEGF	NM001025366	ctacctccaccatgccaagt	tggtgatgttggactcctca
XD	NM000379	acacccaatctgggctacag	ccgggatcttgtaggtgcta

Skeletal muscle fibre properties

We performed histochemical analyses as described in Chapter 4. In brief, we stained serial cryocut cross-sections using the myofibrillar *adenosinetriphosphatase* (mATPase) method according to Guth and Samaha (1970) with minor modifications (Muentener 1979). We classified muscle fibres according to their myosin heavy chain (MYH) isoform into MYH-1, MYH-2A, and MYH-2X, and measured the muscle fibres' diameters by the lesser fibre diameter-method (Dubowitz and Sewry 2007). For all histochemical analyses, we used the NIH Image J software (1.410, National Institutes of Health, Bethesda, MD, USA).

Dual-energy X-ray absorptiometry (DXA)

We used dual-energy X-ray absorptiometry (Lunar iDXA™, GE Healthcare, Madison, WI, USA) to determine whole-body lean and fat masses according to the manufacturer.

Determination of squat 1RM

1RM measurements comprised squatting over the individual's maximal range of motion, usually from near complete extension to a knee angle of slightly less than 90° in 4 s (eccentric action) and back to near complete extension in 4 s (concentric action). Transition from eccentric to concentric action was performed smoothly within 1-2 s. The warm-up was followed by three repetitions at 70% anticipated maximum. Three to five subsequent attempts were made to determine the 1RM, with a 5 min rest interval between squats.

Statistical analyses

Data are presented as mean (SD). To obtain normally distributed data, mRNA copy numbers were log-transformed. Comparisons between groups were performed by one-way analysis of variance (ANOVA). Differences between individual means were tested for statistical significance by *t*-tests for dependent samples with Bonferroni adjustment. The level of significance was set to $P < 0.05$. For all statistical analyses, SPSS 16.0 statistical software (SPSS, Chicago, IL, USA) was used.

Results

Characterisation of study participants

Squat 1RM of study participants was 121 (18) kg. A detailed anthropometric description of study participants is depicted in Table 5.2. For the histochemical analyses a mean of 159 (66) and 122 (54) fibres were counted per muscle biopsy for determination of fibre types and muscle fibre cross-sectional area, respectively.

Table 5.2: Anthropometric data of study participants

Body mass (kg)	75.3 (9.2)
Body height (m)	1.81 (0.04)
Body lean mass (kg)	61.7 (7.7)
Body fat mass (kg)	11.6 (2.3)
Body fat (%)	15.8 (2.4)
Leg lean mass (kg)	21.3 (2.9)
Leg fat mass (kg)	4.2 (1.0)
% MYH-1	63.2 (7.4)
% MYH-2A	24.2 (3.5)
% MYH-2X	12.6 (8.0)
MYH-1 CSA (10-6 m2)	3011 (564)
MYH-2A CSA (10-6 m2)	3321 (588)
MYH-2X CSA (10-6 m2)	2883 (670)

Data are means (SD). MYH, myosin heavy chain isoform; CSA, cross-sectional area.

Skeletal muscle mRNA analyses

Mitochondrial biogenesis. PGC-1α mRNA expression increased significantly to ~ 4.4-fold (P < 0.001) 3 h after a single bout of VRO. In contrast, PGC-1α mRNA abundance increased only ~ 2.4-fold (P = 0.227) after RES (Fig. 5.1A) being significantly different from VRO (P = 0.036). There was no increase observed in transcription factors associated with mitochondrial biogenesis such as ERRα, NRF1, and Tfam after both exercise modalities (Fig. 5.1B, C, and D).

Angiogenesis and oxygen sensing. VEGF mRNA abundance was solely elevated after VRO approximately twofold (P < 0.001), whereas no increase was observed after RES (P = 0.881, group difference P < 0.024, Fig. 5.2A). No changes were detected in HIF-1α and the HIF prolyl hydroxylase 3 (PHD3) mRNA expression after both VRO and RES (Fig. 5.2B and C).

Metabolic key enzymes. HKII mRNA increased 3 h after a single bout of VRO ~ 2.5-fold (P = 0.004) but not after RES (P = 0.715, group difference P = 0.020, Fig. 5.3A). mRNA levels of other metabolic key enzymes such as CS, LDHA, and PFKm were not affected after both exercise modalities (Fig. 5.3B, C, and D)

Oxidative stress. The mRNA abundance of XD increased significantly after VRO (~ 2.3-fold, P = 0.007) but not after RES (P = 0.665) with no group difference (P = 0.632, Fig. 5.4A). Furthermore, MnSOD mRNA abundance only increased after VRO (~ 1.6-fold, P = 0.048) but not after RES (~ 0.8-fold, P = 0.167) being significantly different from VRO (P = 0.001) (Fig. 5.4B).

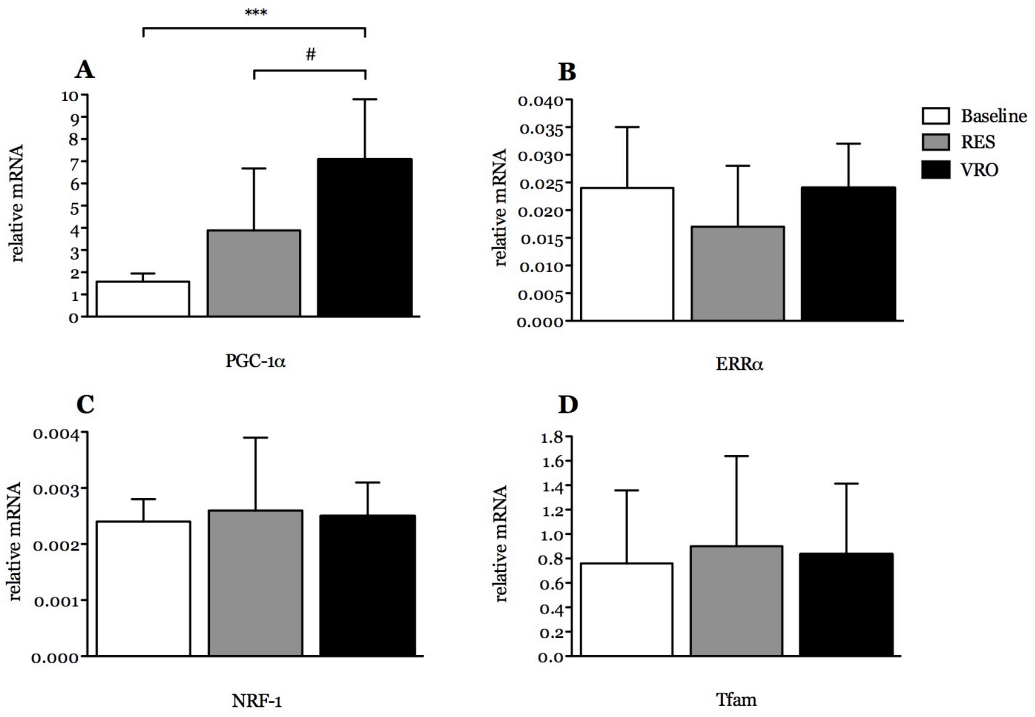

Figure 5.1: Alterations in PGC-1α (A), ERRα (B), NRF-1 (C), and Tfam (D) mRNA abundances 3 h after a single bout of RES (grey bars) or VRO (black bars) relative to baseline (white bars) levels. Bars and error bars represent mean values and standard deviations, respectively. ERRα, estrogen-related receptor α NRF-1, nuclear respiratory factor 1; PGC-1α, peroxisome proliferator-activated receptor coactivator 1α; RES, resistance exercise; Tfam, mitochondrial transcription factor A; VRO, Galileo® vibration + resistance exercise + vascular occlusion. *** significantly different from baseline, P < 0.001; # significantly different within exercise modality RES vs. VRO, P < 0.05.

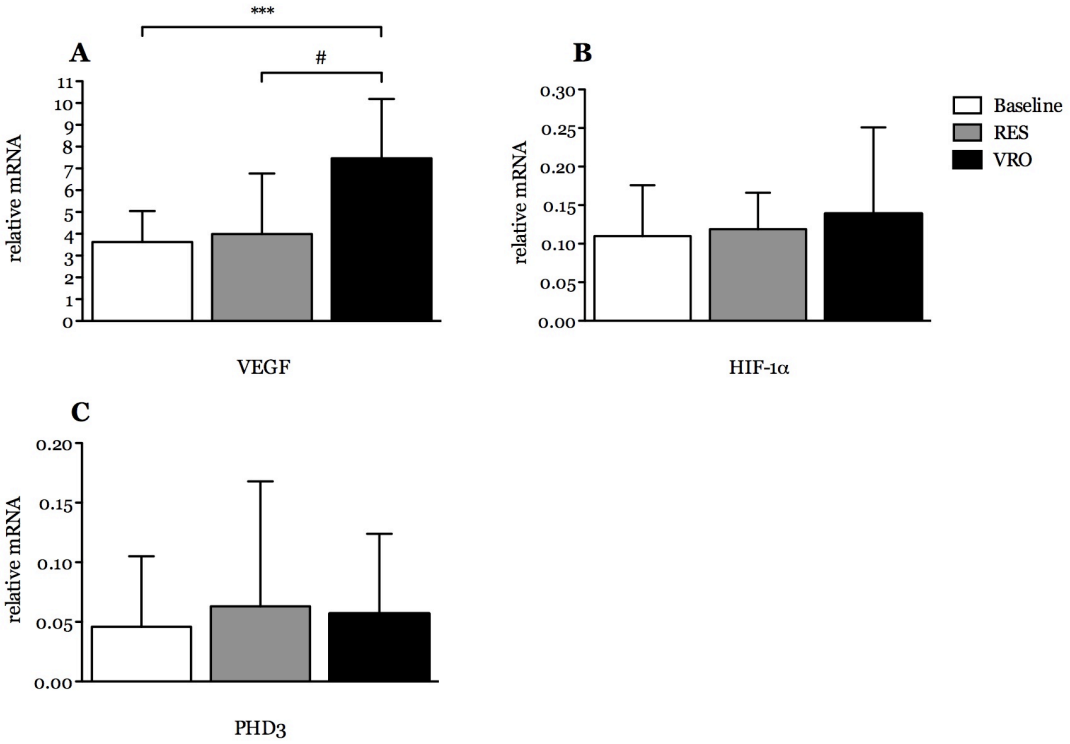

Figure 5.2: Alterations in VEGF (A), HIF-1α (B), and PHD3 (C) mRNA abundances 3 h after a single bout of RES (grey bars) or VRO (black bars) relative to baseline (white bars) levels. Bars and error bars represent mean values and standard deviations, respectively. HIF-1α, hypoxia-inducible transcription factor; PHD3, prolyl hydroxylase dehydrogenase 3; RES, resistance exercise; VEGF, vascular endothelial growth factor; VRO, Galileo® vibration + resistance exercise + vascular occlusion. *** significantly different from baseline, $P < 0.001$; # significantly different within exercise modality RES vs. VRO, $P < 0.05$.

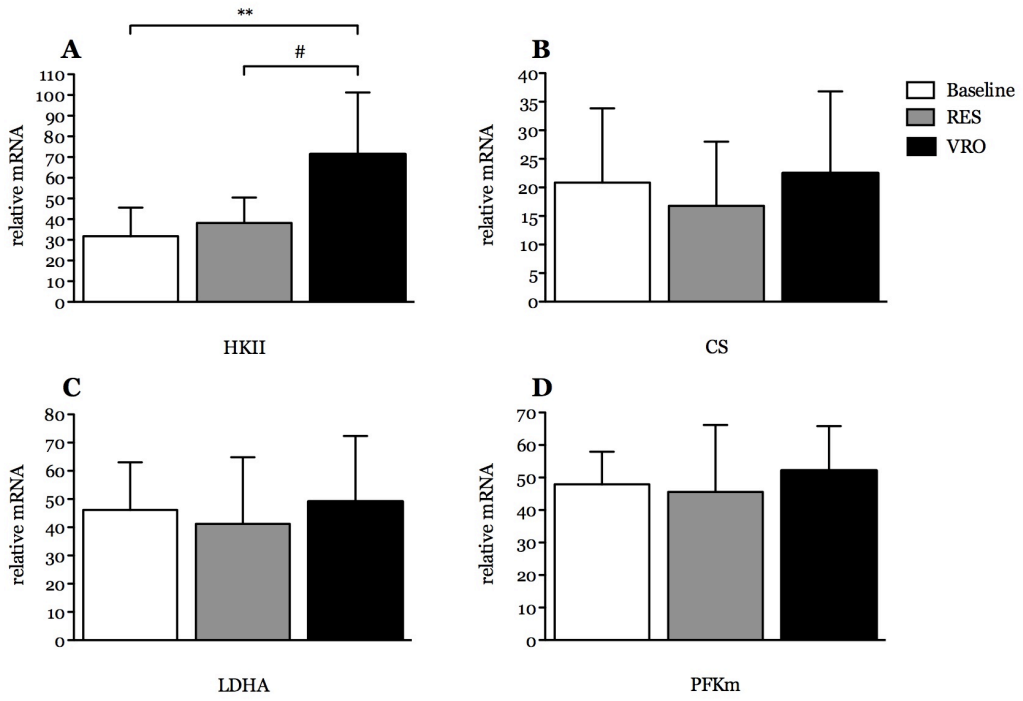

Figure 5.3: Alterations in HKII (A), CS (B), LDHA (C), and PFKm (D) mRNA abundances 3 h after a single bout of RES (grey bars) or VRO (black bars) relative to baseline (white bars) levels. Bars and error bars represent mean values and standard deviations, respectively. CS, citrate synthase; HKII, hexokinase II; LDHA, lactate dehydrogenase A; PFKm, phospho fructokinase, muscle type; RES, resistance exercise; VRO, Galileo® vibration + resistance exercise + vascular occlusion. ** significantly different from baseline, $P < 0.01$; # significantly different within exercise modality RES vs. VRO, $P < 0.05$.

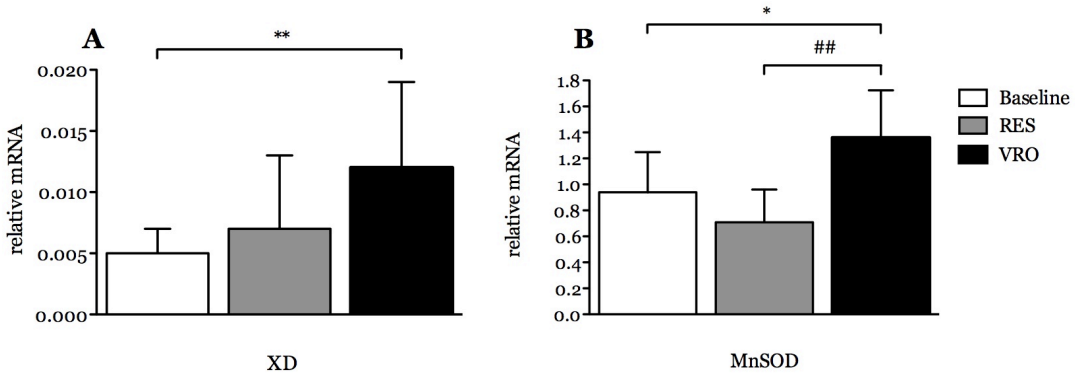

Figure 5.4: Alterations in XD (A) and MnSOD (B) mRNA abundances 3 h after a single bout of RES (grey bars) or VRO (black bars) relative to baseline (white bars) levels. Bars and error bars represent mean values and standard deviations, respectively. MnSOD, manganese superoxide dismutase; RES, resistance exercise; VRO, Galileo® vibration + resistance exercise + vascular occlusion; XD, xanthine dehydrogenase. * significantly different from baseline, $P < 0.05$; ## significantly different within exercise modality RES vs. VRO, $P < 0.01$.

Correlations between alterations in mRNA abundances

The increase of mRNA for PGC-1α was positive correlated with the increase of mRNA for VEGF ($R^2 = 0.653$, $P < 0.001$, Fig. 5.5A) and MnSOD ($R^2 = 0.200$, $P = 0.082$, Fig. 5.5B) after RES and VRO.

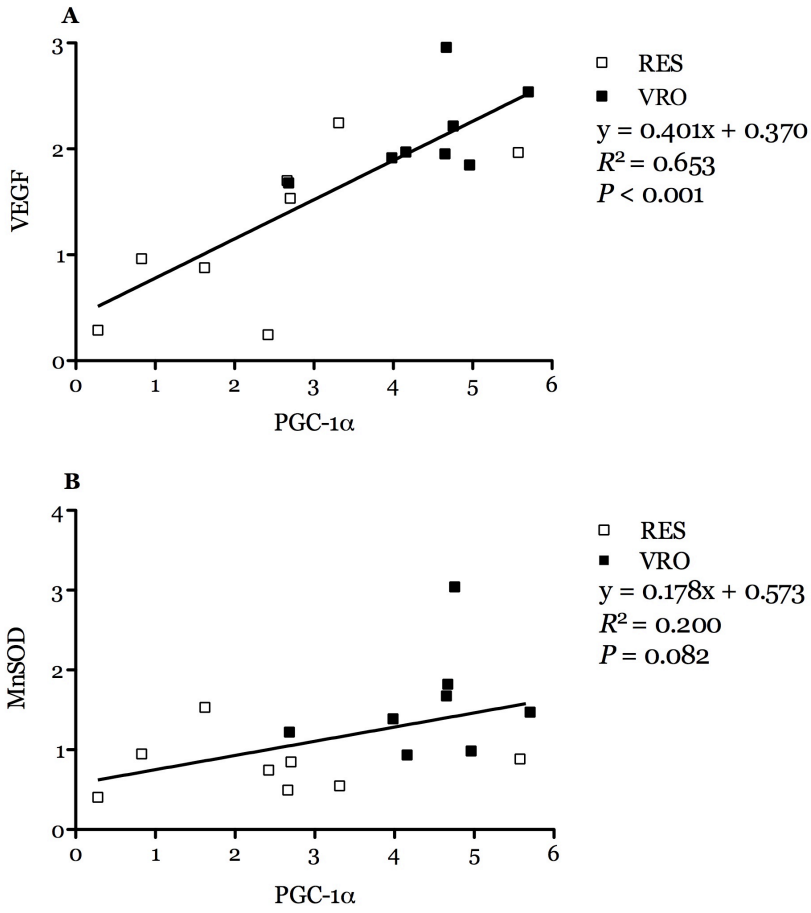

Figure 5.5: Increases of VEGF mRNA (A) and MnSOD mRNA (B) as a function of increases in PGC-1α mRNA abundance after VRO and RES. MnSOD, manganese superoxide dismutase; PGC-1α, peroxisome proliferator-activated receptor coactivator 1α; VEGF, vascular endothelial growth factor.

Discussion

The principal finding of the present study was that two sets of VRO increased both the expression of PGC-1α and VEGF, in contrast to RES alone. Furthermore, only VRO increased HKII, XD, and MnSOD mRNA abundances. These findings indicate

that VRO activates gene programmes normally associated with endurance exercise and/or oxidative stress.

Three hours post-exercise, VEGF and PGC-1α mRNA abundances were increased for VRO but not RES (Fig. 5.1A, 5.2A), indicating that the superimposed stimuli (*i.e.* whole-body vibration and vascular occlusion) mediated the increase in VEGF and PGC-1α expression. VEGF plays an essential role in exercise-induced angiogenesis in skeletal muscle (Olfert et al. 2010), and a reduced oxygen partial pressure represents a major stimulus for inducing VEGF (Forsythe et al. 1996). However, it remains inconclusive as to whether endurance exercise in systemic hypoxia or with restricted blood flow potentiates the VEGF response (Gustafsson et al. 1999, 2002, Richardson 1999). Questioning the importance of a reduced cellular partial oxygen pressure as a stimulus for an exercise-induced increase in VEGF expression Richardson et al. (1999) suggested that there exists an intracellular oxygen partial pressure "threshold" beyond which no greater angiogenic stimulus is produced, and that endurance exercise in normoxia can achieve this threshold. However, vascular occlusion was sustained after exhaustive resistance exercise in VRO leading to a sufficient stimulus to increase the VEGF expression.

In cell culture, it has been shown that hypoxia increases the VEGF mRNA abundance through transcription factor HIF-1 activated VEGF promoter activity (Forsythe et al. 1996). HIF-1 is a heterodimeric transcription factor composed of the HIF-1α and HIF-1β subunits and its activity is post-translationally regulated by HIF-1α stabilisation in hypoxic conditions. Similar to hypoxia, endurance exercise has been shown to induce VEGF mRNA and HIF-1α protein abundances (Ameln et al. 2005, Gustafsson et al. 1999). Notably, HIF-1α mRNA expression in human skeletal muscles remains unaffected with endurance exercise (Ameln et al. 2005, Gustafsson et al. 1999). VRO neither increased HIF-1α mRNA abundance nor the expression of HIF-1α target genes (*e.g.* PHD3, LDHA, PFKa) (Fig. 5.2B, C, 5.3C, D), which leads us to suggest that VEGF expression was induced in a HIF-1 independent manner. Nevertheless, we cannot exclude that the increase in VEGF expression was driven by HIF-1α, since we did not assess HIF-1α protein content or activity.

Instead of HIF-1α, two other possible mechanisms could explain why VEGF was induced after VRO but not RES: 1) increased PGC-1α and/or 2) shear stress. In fact, we found a very strong correlation between PGC-1α and VEGF mRNA expression

(R^2 = 0.653, P < 0.001, Fig. 5.5A). It has been reported that PGC-1α regulates VEGF in rodent skeletal muscles (Chinsomboon et al. 2009, Geng et al. 2010), probably in a HIF-1α independent way (Arany et al. 2008). Recently, it has also been shown that the acute response of PGC-1α mRNA to high-intensity exercise depends on exercise intensity (Egan et al. 2010, Nordsborg et al. 2010) and/or muscle fibre activation pattern (Godin et al. 2010), indicating that high power output and increased metabolic cell stress are key factors for inducing PGC-1α expression. Since PGC-1α expression was increased after VRO only, superimposed sustained vascular occlusion and whole-body vibration might have triggered this response. In fact, (sustained) vascular occlusion further increases metabolic cell stress (*e.g.* accumulation of metabolites and ions) and side-alternating vibration increases power output. Since VRO and RES were both based on high-intensity resistance exercise at 70% of 1RM, leading to a complete motor unit recruitment of the leg muscles when completed to exhaustion (DeLuca 1996), it seems unlikely that a diverse muscle fibre activation pattern accounted for the different PGC-1α expression after VRO and RES.

Concomitant to the increase in PGC-1α expression, we found that MnSOD and XD mRNA abundances were both elevated solely after VRO, which indicates the presence of oxidative stress (Fig. 5.4). Since it has been shown that PGC-1α signalling is sensitive to ROS (Kang et al. 2009, St-Pierre et al. 2006), we suspect that the elevated oxidative stress after VRO led to the observed increase in PGC-1α expression. Consistent with this notion, we found a moderate but barely not significant correlation between PGC-1α and MnSOD mRNA expression (R^2 = 0.200, P = 0.082, Fig. 5.5B). The increase in XD mRNA after VRO but not RES further highlights the specific roles of (sustained) vascular occlusion and vibration in the generation of oxidative stress. XD is the dehydrogenase form of xanthine oxidase (XO), which is localised in the vascular walls of skeletal muscles but not in the skeletal muscle itself (Hellsten-Westing 1993). This fact is highlighted by the low XD mRNA copy numbers (Fig. 5.4A). XO is capable of producing superoxide radicals (Vina et al. 2000) and has been identified as a source of superoxide in reperfusion injury. Thus, it can be speculated that similar to reperfusion injury, VRO led to the conversion of XD to XO in endothelial cells of the vascular walls, and subsequently to the production of ROS, which in turn triggered the expression of PGC-1α in skeletal muscle cells. Alternatively or concomitantly, VEGF expression after VRO might also have been driven by mechanical and shear stresses (Prior et al. 2004). In

this regard, vibration exercise has been reported to induce shear stress at the wall of vessels leading to increases in circulating blood VEGF protein (Suhr et al. 2007). Thus, based on our findings it appears that both sustained vascular occlusion and/or the associated reperfusion, and whole body vibration contributed to the induction of VEGF mRNA abundance after VRO.

Although PGC-1α expression was significantly increased after VRO, ERRα, NRF-1, and Tfam remained unaffected (Fig. 5.2B, C, and D). These findings are in line with reports of other studies in humans, where it has also been observed that despite the marked increase in PGC-1α, NRF1, and Tfam remain unaffected (Norrbom et al. 2004, Pilegaard et al. 2003). In contrast, in C2C12 myotubes overexpressing PGC-1α, NRF1 and NRF2α mRNA are dramatically increased (Wu et al. 1999). Moreover, HKII, a gene that is often associated with adaptations to endurance training, was only up-regulated after VRO (Fig. 5.3A), while other metabolic key enzymes such as CS, LDHA, and PFKm (Fig. 5.3B, C, and D) were not increased after VRO or RES. Pilegaard et al. (2003) also found that HKII is the only metabolic key enzyme whose expression is increased after 3 h of knee extension exercise. These authors suggested that genes display a different responsiveness to exercise and that there exists a temporal hierarchy to the transcriptional activation of some genes after a single exercise stimulus. The fact that the responses of these key metabolic genes in the present study are in line with other investigations supports this concept.

The herein reported findings about the molecular changes after an acute bout of VRO coincide with the observed long-term adaptations following repeated VRO exercise. Indeed, in Chapter 4 we have shown that 5 weeks of VRO training increases capillary-to-fibre ratio, skeletal muscle oxidative enzyme activity, MYH-1 fibre proportion, and endurance capacity. Altogether, these findings indicate that repeated VRO training mediates a shift towards a more oxidative and fatigue resistant muscle. The results of this study now point to a major role of PGC-1α and VEGF in mediating these cellular adaptations. Of special note is, that these VRO-induced changes at the molecular, cellular, and functional levels are typically observed after traditional endurance exercise or more recently also after sprint interval training (Burgomaster et al. 2008, Gibala et al. 2009), but not after resistance exercise. Our findings support previous studies, which disclosed that not the duration of a specific exercise modality is most important for adaptations

leading to an oxidative skeletal muscle fibre phenotype but rather the degree of metabolic perturbation of the skeletal muscles.

In summary, we have reported that modified high-intensity resistance exercise activates gene programmes typically linked to endurance exercise. VRO increased the expression of VEGF mRNA through ROS-activated PGC-1α and probably in a HIF-1α independent manner. Vascular occlusion and increased shear stress probably induced XO in endothelial cells of the vascular walls, which subsequently induced the production of ROS. Whole-body vibration and (sustained) vascular occlusion led to a high metabolic cell stress which further accounts for the presented molecular responses. These results indicate that by the addition of specific stimuli to traditional exercise modalities the molecular response and adaptations can be modified.

Chapter 6

General discussion and outlook

The present thesis was aimed at providing new insights into the mechanisms underlying aerobic function and skeletal muscle plasticity. In order to achieve our objectives, we established two methods (*e.g*. electric pulse stimulation [EPS] cell culture system and magnetic resonance-compatible ergometer setup), designed a new exercise modality (*e.g* side-alternating whole body vibration training with superimposed resistance exercise and vascular occlusion [VRO]), and performed four studies by using cell culture, rodent, and human models.

Typically, improvements in aerobic function result after endurance exercise training, whereas an impaired aerobic function is related to physical inactivity and chronic diseases. In chapter 2 of this thesis, we provided substantial evidence that oxidative capacity and aerobic exercise performance in young untrained women with type 1 diabetes are not reduced compared to healthy women of similar age and activity, but that in women with type 1 diabetes, glycaemic status affects oxidative capacity. These findings reveal that only patients with type 1 diabetes and high HbA1c (> 7.6%) have a reduced oxidative capacity as compared to patients with type 1 diabetes and low HbA1c. However, based on the negative correlation between HbA1c and oxidative capacity, it is not clear whether there exist a causal connection. A recent study reported that endurance athletes (following an endurance exercise training programme) with type 1 diabetes and high (> 7%) HbA1c have lower peak oxygen consumption, peak stroke volume, and peak cardiac output relative to athletes with low (< 7%) HbA1c, and that HbA1c negatively correlates with peak stroke volume

(Baldi et al. 2010). Although in the study of Baldi et al. (2010) only systemic factors affecting aerobic function were analysed and not oxidative capacity, these results support our findings that glycaemic status makes a major impact on aerobic function. The practical relevance of these findings is that in order to gain health benefits, patients with type 1 diabetes are advised first to lower their glycaemic status, and then second to improve their aerobic function by regular physical activity. Furthermore, it is important to accurately control glycaemic status in future studies investigating the aerobic function in patients with type 1 diabetes. It is possible that although participants in our study were untrained (less than 1 h of physical activity per week), it could be that the patients with lower HbA1c levels rather follow a moderate physically active life-style (*e.g.* walking and cycling instead of taking public transport or car, using the elevator), whereas participants with higher HbA1c levels are entirely physical inactive. If this is true, environmental/ lifestyle-related factors would account for the reduced oxidative capacity rather then the level of glycaemic control. Nevertheless, based on the fact that we and Baldi et al. (2010) accurately controlled the physical activity behaviour of the study participants it is very likely that glycaemic status makes a major impact on aerobic function.

We developed a MR-compatible ergometer setup which was comprised of a dynamometer with an integrated strain gauge for force measurements and a real-time visual feedback system for providing the participants with information on the level of exerted force relative to maximal voluntary force. The combination of this ergometer setup with ^{31}P-MRS allowed us to measure real-time skeletal muscle energetic properties at rest, during, and after exercise. We propose to use this tool in future studies to analyse the oxidative capacity of patients with a broader range of HbA1c levels and physical activity behaviour. Then, these studies should provide more information about the roles of physical activity and aerobic function in the aetiology of diabetes. A further approach to elucidate the molecular and cellular mechanisms underlying the relationship between glycaemic status and aerobic function of the skeletal muscle is to use our newly developed *in vitro* exercise system. We established a cell culture model using murine C2C12 myocytes that closely recapitulates the plastic changes in gene expression as observed in aerobically trained skeletal muscle of mice (Chapter 3). Thus, we presented evidence that EPS is a valid tool to mimic exercise conditions *in vitro*. In future studies, our EPS system can be extended with additional stimuli such as mechanical stretch and hypoxia. Furthermore, C2C12 myocytes could be replaced by mouse or human

primary myoblasts. By using primary myoblasts from donors with low or high HbA1c levels instead of C2C12 myocytes, this system would be an elegant tool to investigate the molecular and cellular mechanism underlying the relationship between glycaemic status and aerobic function of the skeletal muscle in relation to physical activity.

To determine whether it is possible to improve aerobic function in humans with an exercise modality which is based on resistance exercise, we aimed at examining the effectiveness and efficiency of VRO. We presented evidence that VRO training led to a more oxidative skeletal muscle phenotype (Chapter 4) which was additionally supported by the finding that a single bout of VRO activated gene programmes normally associated with endurance exercise (Chapter 5). As a next step, the single stimuli of VRO need to be analysed in order to assign a specific adaptation/effect to a specific stimulus. However, being aware of the very short time commitment of VRO which led to the relevant adaptations/effects, it is likely that the combination of the different stimuli rather than a single stimulus was responsible for the observed outcomes. The substantial findings presented in our human studies are in line with recent reports that not only traditional endurance exercise training leads to a more oxidative skeletal muscle phenotype and an associated improved endurance capacity but also high-intensity cycling and sprint interval training (Burgomaster et al. 2008, Egan et al. 2010, Gibala et al. 2009, Nordsborg et al. 2010). Our results support the notion that not the duration of a specific exercise modality is most important for adaptations leading to an oxidative skeletal muscle fibre phenotype but rather the degree of metabolic perturbation of the skeletal muscles. Our *in vitro* exercise system could further be useful to deepen our understanding of the exact molecular mechanism underlying the adaptations to VRO. As VRO was comprised of vibration and resistance exercise with superimposed vascular occlusion, these stimuli are present in our proposed *in vitro* exercise system. Thus, this system would be a valuable tool to investigate the single and additive effects of hypoxia, cyclic stretch, and EPS and would serve as a valuable tool which complements our human studies.

As we initially proposed, it is fundamental to identify which mechano-biological condition leads to the molecular/cellular response that is associated with an oxidative gene programme, and how this molecular/cellular response relates to an improved aerobic function (Chapter 1, Toigo and Boutellier 2006). The results presented in this thesis nicely illustrate that by addition of specific stimuli to

traditional exercise modalities (*e.g.* endurance or resistance exercise) the molecular response and adaptations can be modified and thus be analysed. We demonstrated a possibility for future studies to investigate the importance of specific exercise stimuli. The future challenge is to fully elucidate the contribution of each single exercise stimuli to the exercise-induced adaptations and effects. With this knowledge we will be able to design new effective therapeutic exercise interventions for many of the chronic diseases.

Literature

Akimoto T, Pohnert S, Li P, Zhang M, Gumbs C, Rosenberg P, Williams R, Yan Z (2005) Exercise stimulates PGC-1 alpha transcription in skeletal muscle trough activation of the p38 MAPK pathway. J Biol Chem 280:19587-19593

Andersen P, Henriksson J (1977) Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. J Physiol 270:677-690

Ameln H, Gustafsson T, Sundberg CJ, Okamoto K, Jansson E, Poellinger L, Makino Y (2005) Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. FASEB J 19:1009-1011

American Diabetes Association: Diagnosis and classification of diabetes mellitus (Position statement) (2006) Diabetes Care 1:S43-S48

American Diabetes Association: Standards of medical care in diabetes – 2008 (Position statement) (2008) Diabetes Care 1:S12-S54

Arany Z, Foo S-Y, Ma Y, Ruas JL, Bommi-Reddy A, Gimun G, Cooper M, Laznik D, Chinsomboon J, Rangwala SM, Baek KH, Rosenzweig A, Spiegelman BM (2008) HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1 alpha. Nature 451:1008-1012

Arnold DL, Matthews PM, Radda GK (1984) Metabolic recovery after exercise and the assessment of mitochondrial function in vivo in human skeletal muscle by means of 31P NMR. Magn Reson Med 1:307-315

Atherton PJ, Babraj J, Smith K, Singh J, Rennie MJ, Wackerhage H (2005) Selective activation of AMPK-PGC-1 alpha or PKB-TSC2-mTOR signalling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation. FASEB J 19:786-788

Baar K (2006) Training for endurance and strength: lessons from cell signalling. Med Sci Sports Exerc 38:1939-1944

Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, Holloszy JO (2002) Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J 16:1879-1886

Baldi JC, Cassuto NA, Foxx-Lupo WT, Wheatley CM, Snyder EM (2010) Glycaemic status affects cardiopulmonary exercise response in athletes with type 1 diabetes. Med Sci Sports Exerc 42:1454-1459

Baldwin KM, Haddad F (2002) Skeletal muscle plasticity: cellular and molecular responses to altered physical activity paradigms. Am J Phys Med Rehabil 81:S40-S51

Booth FW, Baldwin KM (1995) Muscle plasticity: energy demanding and supply processes. In: Rowell LB, Sheperd JT (eds.) Handbook of physiology, Oxford University Press, New York, USA, pp 1076-1121

Booth FW, Chakravarthy MV, Gordon SE, Spangenburg EE (2002) Waging war on physical inactivity: using modern molecular ammunition against an ancient enemy. J Appl Physiol 93:3-30

Booth FW, Laye MJ (2009) Lack of adequate appreciation of physical exercise's complexities can pre-empt appropriate design and interpretation in scientific discovery. J Physiol 587:5527-5539

Booth FW, Laye MJ, Lees SJ, Rector RS, Thyfault JP (2008) Reduced physical activity and risk of chronic disease: the biology behind the consequences. Eur J Appl Physiol 102:381-390

Booth FW, Lees SJ (2007) Fundamental questions about genes, inactivity, and chronic diseases. Physiol Genomics 28:146-157

Booth FW, Thomason DB (1991) Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models. Physiol Rev 71:541-585

Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsoe R, Dela F (2007) Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. Diabetologia 50:790-796

Brooks GA, Fahey TD, Baldwin KM (2005) Skeletal muscle structure and contractile properties. In: Exercise physiology - Human bioenergetics and its applications. 4th edn. McGraw Hill Companies, New York, USA, pp 363-395

Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, Macdonald MJ, McGee SL, Gibala MJ (2008) Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. J Physiol 586:151-160

Burgomaster KA, Moore DR, Schofield LM, Phillips SM, Sale DG, Gibala MJ (2003) Resistance training with vascular occlusion: metabolic adaptations in human muscle. Med Sci Sports Exerc 35:1203-1208

Burnley M (2009) Estimation of critical torque using intermittent isometric maximal voluntary contractions of the quadriceps in humans. J Appl Physiol 106:975-983

Caiozzo VJ, Herrick RE, Baldwin KM (1991) The influence of hyperthyroidism on the maximal shortening velocity and myosin isoform distribution in slow and fast skeletal muscle. Am J Physiol 261:C285-C295

Cairns SP, Chin ER, Renaud JM (2007) Stimulation pulse characteristics and electrode configuration determine site of excitation in isolated mammalian skeletal muscle: implications for fatigue. J Appl Physiol 103:359-368

Calvo JA, Daniels TG, Wang X, Paul A, Lin J, Spiegelman BM, Stevenson SC, Rangwala SM (2008) Muscle-specific expression of PPAR gamma coactivator-1 alpha improves exercise performance and increases peak oxygen uptake. J Appl Physiol 104:1304-1312

Campos GE, Luecke TJ, Wendeln HK, Toma K, Hagerman FC, Murray TF, Ragg KE, Ratamess NA, Kraemer WJ, Staron RS (2002) Muscular adaptations in response to three different resistance-training regimens: specificity of repetition maximum training zones. Eur J Appl Physiol 88:50-60

Cardinale M, Lim J (2003) Electromyography activity of vastus lateralis muscle during whole-body vibrations of different frequencies. J Strength Cond Res 17:621-624

Carey DG, Jenkins AB, Campbell LV, Freund J, Chisholm DJ (1996) Abdominal fat and insulin resistance in normal and overweight women: direct measurements reveal a strong relationship in subjects at both low and high risk of NIDDM. Diabetes 45:633-638

Carmeliet P (2000) Mechanism of angiogenesis and arteriogenesis. Nat Med 6:389-395

Centers for Disease Control and Prevention. Physical activity for everyone: the importance of physical activity (2006) Available at: http://www.cdc.gov/ /nccdphp/dnpa/phyisical/importance/index.htm

Chesley A, MacDougall JD, Tarnopolsky MA, Atkinson SA, Smith K (1992) Changes in human muscle protein synthesis after resistance exercise. J Appl Physiol 73:1383-1388

Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R, Williams RS (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fibre type. Genes Dev 12:2499-2509

Chinsomboon J, Ruas J, Gupta RK, Thom R, Shoag J, Rowe GC, Sawada N, Raghuram S, Arany Z (2009) The transcriptional coactivator PGC-1 alpha mediates exercise-induced angiogenesis in skeletal muscle. Proc Natl Acad Sci USA 106:21401-21406

Choi S, Liu X, Li P, Akimoto T, Lee SY, Zhang M, Yan Z (2005) Transcriptional profiling in mouse skeletal muscle following a single bout of voluntary running: evidence of increased cell proliferation. J Appl Physiol 99:2406-2415

Chow LS, Greenlund LJ, Asmann YW, Short KR, McCrady SK, Levine JA, Nair KS (2007) Impact of endurance training on murine spontaneous activity, muscle mitochondrial DNA abundance, gene transcripts, and function. J Appl Physiol 102:1078-1089

Coffey VG, Hawley JA (2006) Training for performance: insights from molecular biology. Int J Sports Physiol Perform 1:284-292

Coffey VG, Hawley JA (2007) The molecular bases of training adaptation. Sports Med 37:737-763

Coffey VG, Shield A, Canny BJ, Carey KA, Cameron-Smith D, Hawley JA (2006) Interaction of contractile activity and training history on mRNA abundance in skeletal muscle from trained athletes. Am J Physiol Endocrinol Metab 290:E849-E855

Conley KE, Jubrias SA, Esselman PC (2000) Oxidative capacity and ageing in human muscle. J Physiol 526:203-210

Consitt LA, Bell JA, Houmard JA (2009) Intramuscular lipid metabolism, insulin action, and obesity. IUBMB Life 61:47-55

Constable SH, Favier RJ, McLane JA, Fell RD, Chen M, Holloszy JO (1987) Energy metabolism in contracting rat skeletal muscle: adaptation to exercise training. Am J Physiol 253:C316-C322

Corpeleijn E, Saris WH, Blaak EE (2009) Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle. Obes Rev 10:178-193

Crowther GJ, Milstein JM, Jubrias SA, Kushmerick MJ, Gronka RK, Conley KE (2003) Altered energetic properties in skeletal muscle of men with well-controlled insulin-dependent (type 1) diabetes. Am J Physiol Endocrinol Metab 284:E655-E662

Dabelea D, Kinney G, Snell-Bergeon JK, Hokanson JE, Eckel RH, Ehrlich J, Garg S, Hamman RF, Rewers M (2003) Effect of type 1 diabetes on the gender difference in coronary artery calcification: a role for insulin resistance? Diabetes 52:2833-2839

De Luca CJ, Foley PJ, Erim Z (1996) Motor unit control properties in constant-force isometric contractions. J Neurophysiol 76:1503-1516

DeFronzo RA, Hendler R, Simonson D (1982a) Insulin resistance is a prominent feature of insulin-dependent diabetes. Diabetes 31:795-801

DeFronzo RA, Simonson D, Ferrannini E (1982b) Hepatic and peripheral insulin resistance: a common feature of type 2 (non-insulin-dependent) and type 1 (insulin-dependent) diabetes mellitus. Diabetologia 23:313-319

Delmonico MJ, Kostek MC, Johns J, Hurley BF, Conway JM (2008) Can dual energy X-ray absorptiometry provide a valid assessment of changes in thigh muscle mass with strength training in older adults? Eur J Clin Nutr 62:1372-1378

Donath MY, Halban PA (2004) Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. Diabetologia 47:581-589

Drexler H, Riede U, Munzel T, Konig H, Funke E, Just H (1992) Alterations of skeletal muscle in chronic heart failure. Circulation 85:1751-1759

Dubowitz V, Sewry CA (2007) Muscle Biopsy. 3rd edn. Saunders Elsevier, pp 83-84

Dufour E, Larsson NG (2004) Understanding ageing: revealing order out of chaos. Biochim Biophys Acta 23:122-132

Dunn SE, Burns JL, Michel RN (1999) Calcineurin is required for skeletal muscle hypertrophy. J Biol Chem 274:21908-21912

Duscha BD, Kraus WE, Keteyian SJ, Sullivan MJ, Green HJ, Schachat FH, Pippen AM, Brawner CA, Blank JM, Annex BH (1999) Capillary density of skeletal muscle: a contributing mechanism for exercise intolerance in class II-III chronic heart failure independent of other peripheral alterations. J Am Coll Cardiol 33:1956-1963

Ebeling P, Koistinen HA, Koivisto VA (1998) Insulin-independent glucose transport regulates insulin sensitivity. FEBS Lett 436:301-303

Egan B, Carson BP, Garcia-Roves PM, Chibalin AV, Sarsfield FM, Barron N, McCaffrey N, Moyna NM, Zierath JR, O'Gorman DJ (2010) Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor coactivator-1 mRNA abundance is associated with differential activation of upstream signaling kinases in human skeletal muscle. J Physiol 588:1779-1790

Egginton S (2009) Invited review: activity-induced angiogenesis. Pflugers Arch 457:963-977

Egginton S (2010) Angiogenesis - may the force be with you. J Physiol 588:4615-4616

Eiken O, Sundberg CJ, Esbjoernsson M, Nygren A, Kaijser L (1991) Effects of ischaemic training on force development and fibre-type composition in human skeletal muscle. Clin Physiol 11:41-49

Esposito F, Mathieu-Costello O, Shabetai R, Wagner PD, Richardson RS (2010) Limited maximal exercise capacity in patients with chronic heart failure: partitioning the contributors. J Am Coll Cardiol 55:1945-1954

Figueiredo PA, Mota MP, Appell HJ, Duarte JA (2008) The role of mitochondria in ageing of skeletal muscle. Biogerontology 9:67-84

Finck BN, Kelly DP (2006) PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin Invest 116:615-622

Fitzsimons DP, Diffee GM, Herrick RE, Baldwin KM (1990) Effects of endurance exercise on isomyosin patterns in fast- and slow-twitch skeletal muscles. J Appl Physiol 68:1950-1955

Flueck M (2006) Functional, structural and molecular plasticity of mammalian skeletal muscle in response to exercise stimuli. J Exp Biol 209:2239-2248

Flueck M, Hoppeler H (2003) Molecular basis of skeletal muscle plasticity-from gene to form and function. Rev Physiol Biochem Pharmacol 146:159-216

Fontana P, Boutellier U, Toigo M (2009) Reliability of measurements with Innocor during exercise. Int J Sports Med 30:747-753

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16:4604-4613

Fritzsche K, Bluher M, Schering S, Buchwalow IB, Kern M, Linke A, Oberbach A, Adams V, Punkt K (2008) Metabolic profile and nitric oxide synthase expression of skeletal muscle fibres are altered in patients with type 1 diabetes. Exp Clin Endocrinol Diabetes 116:606-613

Fujita H, Nedachi T, Kanzaki M (2007) Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes. Exp Cell Res 313:1853-1865

Geng T, Li P, Okutsu M, Yin X, Kwek J, Zhang M (2010) PGC-1 alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fibre-type transformation in mouse skeletal muscle. Am J Physiol Cell Physiol 298:C572-C579

Gettman LR, Ayres JJ, Pollock ML, Jackson A (1978) The effect of circuit weight training on strength, cardiorespiratory function, and body composition of adult men. Med Sci Sports 10:171-176

Gibala MJ, McGee SL, Garnham AP, Howlett KF, Snow RJ, Hargreaves M (2009) Brief intense interval exercise activates AMPK and p38 MAPK signalling and increases the expression of PGC-1 alpha in human skeletal muscle. J Appl Physiol 106:929-934

Glass DJ (2005) Skeletal muscle hypertrophy and atrophy signalling pathways. Int J Biochem Cell Biol 37:1974-1984

Godin R, Ascah A, Daussin FN (2010) Intensity-dependent activation of intracellular signalling pathways in skeletal muscle: role of fibre type recruitment during exercise. J Physiol 588:4073-4074

Goodpaster BH, Thaete FL, Simoneau JA, Kelley DE (1997) Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivity independently of visceral fat. Diabetes 46:1579-1585

Gordon T, Pattullo M (1993) Plasticity of muscle fibre and motor unit types. Exerc Sports Sci Rev 21:331-362

Goto M, Terada S, Kato M, Katoh M, Yokozeki T, Tabata I, Shimokawa T (2000) cDNA cloning and mRNA analysis of PGC-1 in epitrochlearis muscle in swimming-exercised rats. Biochem Biophys Res Commun 274:350-354

Greenbaum CJ (2002) Insulin resistance in type 1 diabetes. Diabetes Metab Res Rev 18:192-200

Gurd BJ, Yoshida Y, Lally J, Holloway GP, Bonen A (2009) The deacetylase enzyme SIRT1 is not associated with oxidative capacity in rat heart and skeletal muscle and its overexpression reduces mitochondrial biogenesis. J Physiol 587:1817-1828

Gusso S, Hofman P, Lalande S, Cutfield W, Robinson E, Baldi JC (2008) Impaired stroke volume and aerobic capacity in female adolescents with type 1 and type 2 diabetes mellitus. Diabetologia 51:1317-1320

Gustafsson T, Knutsson A, Puntschart A, Kaijser L, Nordqvist AC, Sundberg CJ, Jansson E (2002) Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. Pflugers Arch 444:752-759

Gustafsson T, Puntschart A, Kaijser L, Jansson E, Sundberg CJ (1999) Exercise induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am J Physiol 276:H679-H685

Guth L, Samaha FJ (1970) Procedure for the histochemical demonstration of actomyosin ATPase. Exp Neurol 28:365-367

Handschin C (2009) The biology of PGC-1 alpha and its therapeutic potential. Trends Pharmacol Sci 30:322-329

Handschin C (2010) Regulation of skeletal muscle cell plasticity by the peroxisome proliferator-activated receptor gamma coactivator 1 alpha. J Recept Signal Transduct Res 30:376-384

Handschin C, Chin S, Li P, Liu F, Maratos-Flier E, Lebrasseur NK, Yan Z, Spiegelman BM (2007) Skeletal muscle fibre-type switching, exercise intolerance, and myopathy in PGC-1 alpha muscle-specific knock-out animals. J Biol Chem 282:30014-30021

Handschin C, Choi CS, Chin S, Kim S, Kawamori D, Kurpad AJ, Neubauer N, Hu J, Mootha VK, Kim YB, Kulkarni RN, Shulman GI, Spiegelman BM (2007) Abnormal glucose homeostasis in skeletal muscle-specific PGC-1 alpha knockout mice reveals skeletal muscle-pancreatic beta cell crosstalk. J Clin Invest 117:3463-3474

Handschin C, Kobayashi YM, Chin S, Seale P, Campbell KP, Spiegelman BM (2007) PGC-1 alpha regulates the neuromuscular junction programme and ameliorates duchenne muscular dystrophy. Genes Dev 21:770-783

Handschin C, Rhee J, Lin J, Tarr PT, Spiegelman BM (2003) An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1 alpha expression in muscle. Proc Natl Acad Sci USA 100:7111-7116

Handschin C, Spiegelman BM (2006) PGC-1 coactivators, energy homeostasis, and metabolism. Endocr Rev 27:728-735

Handschin C, Spiegelman BM (2008) The role of exercise and PGC1 alpha in inflammation and chronic disease. Nature 454:463-469

Harmer AR, Chisholm DJ, McKenna MJ, Hunter SK, Ruell PA, Naylor JM, Maxwell LJ, Flack JR (2008) Sprint training increases muscle oxidative metabolism during high-intensity exercise in patients with type 1 diabetes. Diabetes Care 31:2097-2102

Hawley JA (2009) Molecular responses to strength and endurance training: are they incompatible? Appl Physiol Nutr Metab 34:355-361

Hawley JA, Holloszy JO (2009) Exericse: it's the real thing! Nutr Rev 67:172-178

Hawley JA, Lessard SJ (2007) Mitochondrial function: use it or lose it. Diabetologia 50:699-702

He J, Watkins S, Kelley DE (2001) Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fibre type in type 2 diabetes and obesity. Diabetes 50:817-823

Hellsten-Westing Y (1993) Immunohistochemical localisation of xanthine oxidase in human cardiac and skeletal muscle. Histochemistry 100:215-222

Hickson RC (1980) Interference of strength development by simultaneously training for strength and endurance. Eur J Appl Physiol Occup Physiol 45:255-263

Hoh JF (1991) Myogenic regulation of mammalian skeletal muscle fibres. News Physiol Sci 6:1-6

Holloszy JO (2009) Skeletal muscle "mitochondrial deficiency" does not mediate insulin resistance. Am J Clin Nutr 89:S463-S466

Holloszy JO, Booth FW (1976) Biochemical adaptations to endurance exercise in muscle. Annu Rev Physiol 38:273-291

Holloszy JO, Coyle EF (1984) Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J Appl Physiol 56:831-838

Holloway GP (2009) Mitochondrial function and dysfunction in exercise and insulin resistance. Appl Physiol Nutr Metab 34:440-446

Hood DA (2001) Invited review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. J Appl Physiol 90:1137-1157

Hood DA, Irrcher I, Ljubicic V, Joseph AM (2006) Coordination of metabolic plasticity in skeletal muscle. J Exp Biol 209:2265-2275

Howald H (1982) Training-induced morphological and functional changes in skeletal muscle. Int J Sports Med 3:1-12

Huang YC, Dennis RG, Baar K (2006) Cultured slow vs. fast skeletal muscle cells differ in physiology and responsiveness to stimulation. Am J Physiol Cell Physiol 291:C11- C17

Huss JM, Torra IP, Staels B, Giguere V, Kelly DP (2004) Estrogen-related receptor alpha directs peroxisome proliferator-activated receptor alpha signalling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. Mol Cell Biol 24:9079-9091

Huxley A (1988) Muscular contraction. Annu Rev Physiol 50:1-16

Inoki K, Zhu T, Guan KL (2003) TSC2 mediates cellular energy response to control cell growth and survival. Cell 115:577-590

Ivy JL (1991) Muscle glycogen synthesis before and after exercise. Sports Med 11:6-19

Jaeger S, Handschin C, St-Pierre J, Spiegelman BM (2007) AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1 alpha. Proc Natl Acad Sci USA 104:12017-12022

Jakobsson P, Jorfeldt L, Henriksson J (1995) Metabolic enzyme activity in the quadriceps femoris muscle in patients with severe chronic obstructive pulmonary disease. Am J Respir Crit Care Med 151:374-377

Jobin J, Maltais F, Doyon JF, LeBlanc P, Simard PM, Simard AA, Simard C (1998) Chronic obstructive pulmonary disease: capillarity and fibre-type characteristics of skeletal muscle. J Cardiopulm Rehabil 18:432-437

Jones AM, Vanhatalo A, Burnley M, Morton RH, Poole DC (2010) Critical power: implications for the determination of VO2max and exercise tolerance. Med Sci Sports Exerc doi:10.1249/MSS.0b013e3181d9cf7f28

Jorgensen S, Wojtaszewski JF, Viollet B, Andreelli F, Birk JB, Hellsten Y, Schjerling P, Vaulont S, Neufer PD, Richter EA, Pilegaard H (2005) Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. FASEB J 19:1146-1148

Josse AR, Tang JE, Tarnopolsky MA, Phillips SM (2010) Body composition and strength changes in women with milk and resistance exercise. Med Sci Sports Exerc 42:1122-1130

Kang C, O'Moore KM, Dickman JR, Ji LL (2009) Exercise activation of muscle peroxisome proliferator-activated receptor-gamma coactivator-1 alpha signalling is redox sensitive. Free Radic Biol Med 47:1394-1400

Katzmarzyk PT, Janssen I (2004) The economic costs associated with physical inactivity and obesity in Canada: an update. Can J Appl Physiol 29:90-115

Kelley DE, He J, Menshikova EV, Ritov VB (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 51:2944-2950

Knutti D, Kressler D, Kralli A (2001) Regulation of the transcriptional coactivator PGC-1 via MAPK-sensitive interaction with a repressor. Proc Natl Acad Sci USA 98:9713-9718

Kraus WE, Torgan CE, Duscha BD, Norris J, Brown SA, Cobb FR, Bales CW, Annex BH, Samsa GP, Houmard JA, Slentz CA (2001) Studies of a targeted risk reduction intervention through defined exercise (STRRIDE). Med Sci Sports Exerc 33:1774-1784

Krogh-Madsen R, Thyfault JP, Broholm C, Mortensen OH, Olsen RH, Mounier R, Ploomgaard P, van Hall G, Booth FW, Pedersen BK (2010) A 2-wk reduction of ambulatory activity attenuates peripheral insulin sensitivity. J Appl Physiol 108:1034-1040

Larsson H, Daugaard JR, Kiens B, Richter EA, Ahren B (1999) Muscle fibre characteristic in postmenopausal women with normal or impaired glucose tolerance. Diabetes Care 22:1330-1338

Lee WJ, Kim M, Park HS, Kim HS, Jeon MJ, Oh KS, Koh EH, Won JC, Kim MS, Oh GT, Yoon M, Lee KU, Park JY (2006) AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPAR alpha and PGC-1. Biochem Biophys Res Commun 340:291-295

Leick L, Hellsten Y, Fentz J, Lyngby SS, Wojtaszewski JFP, Hidalgo J, Pilegaard H (2009) PGC-1 alpha mediates exercise-induced skeletal muscle VEGF expression in mice. Am J Physiol Endocrinol Metab 297:E92-E103

Lin J, Handschin C, Spiegelman BM (2005) Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab 1:361-370

Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 418:797-801

Linari M, Bottinelli R, Pellegrino MA, Reconditi M, Reggiani C, Lombardi V (2004) The mechanism of the force response to stretch in human skinned muscle fibres with different myosin isoforms. J Physiol 554:335-352

Lowell BB, Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. Science 307:384-387

Mador MJ, Bozkanat E (2001) Skeletal muscle dysfunction in chronic obstructive pulmonary disease. Respir Res 2:216-224

Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA (2005) Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. FASEB J 19:1498-1500

Marotta M, Bragos R, Gomez-Foix AM (2004) Design and performance of an electrical stimulator for long-term contraction of cultured muscle cells. Biotechniques 36:68-73

Martel GF, Roth SM, Ivey FM, Lemmer JT, Tracy BL, Hurlbut DE, Metter EJ, Hurley BF, Rogers MA (2006) Age and sex affect human muscle fibre adaptations to heavy-resistance strength training. Exp Physiol 91:457-464

Matsuoka Y, Inoue A (2008) Controlled differentiation of myoblast cells into fast and slow muscle fibres. Cell Tissue Res 332:123-132

Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28:412-419

McCall GE, Byrnes WC, Dickinson AL, Fleck SJ (1998) Sample size required for the accurate determination of fibre area and capillarity of human skeletal muscle. Can J Appl Physiol 23:594-599

McGarry JD (2002) Banting lecture 2001: dysregulation of fatty acid metabolism in the aetiology of type 2 diabetes. Diabetes 51:7-18

Meyer RA (1988) A linear model of muscle respiration explains monoexponential phosophocreatine changes. Am J Physiol 254:C548-C553

Mootha VK, Handschin C, Arlow D, Xie X, St-Pierre J, Sihag S, Yang W, Altshuler D, Puigserver P, Patterson N, Willy PJ, Schulman IG, Heyman RA, Lander ES, Spiegelman BM (2004) Err alpha and Gabpa/b specify PGC-1 alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. Proc Natl Acad Sci USA 101:6570-6575

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC (2003) PGC-1 alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 34:267-273

Morino K, Petersen KF, Shulman GI (2006) Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. Diabetes 55:S9-S15

Muentener M (1979) Variable pH dependence of the myosin-ATPase in different muscles of the rat. Histochemistry 62:299-304

Murgia M, Serrano AL, Calabria E, Pallafacchina G, Lomo T, Schiaffino S (2000) Ras is involved in nerve-activity-dependent regulation of muscle genes. Nat Cell Biol 2:142-147

Nadeau KJ, Regensteiner JG, Bauer TA, Brown MS, Dorosz JL, Hull A, Zeitler P, Draznin B, Reusch JE (2010) Insulin resistance in adolescents with type 1 diabetes and its relationship to cardiovascular function. J Clin Endocrinol Metab 95:513-521

Nader GA (2005) Molecular determinants of skeletal muscle mass: getting the “AKT” together. Int J Biochem Cell Biol 37:1985-1996

Nader GA (2006) Concurrent strength and endurance training: from molecules to man. Med Sci Sports Exerc 38:1965-1970

Naumann K, Pette D (1994) Effects of chronic stimulation with different impulse patterns on the expression of myosin isoforms in rat myotube cultures. Differentiation 55:203-211

Nedachi T, Fujita H, Kanzaki M (2008) Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. Am J Physiol Endocrinol Metab 295:E1191-E1204

Nedachi T, Hatakeyama H, Kono T, Sato M, Kanzaki M (2009) Characterisation of contraction-inducible CXC chemokines and their roles in C2C12 myocytes. Am J Physiol Endocrinol Metab 297:E866-E878

Nielsen JN, Richter EA (2003) Regulation of glycogen synthase in skeletal muscle during exercise. Acta Physiol Scand 178:309-319

Nordsborg NB, Lundby C, Leick L, Pilegaard H (2010) Relative workload determines exercise-induced increases in PGC-1 alpha mRNA. Med Sci Sports Exerc 42:1477-1484

Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, Gustafsson T (2004) PGC-1 alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. J Appl Physiol 96:189-194

Olfert IM, Howlett RA, Tang K, Dalton ND, Gu Y, Peterson KL, Wagner PD, Breen EC (2009) Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. J Physiol 587:1755-1767

Olfert IM, Howlett RA, Wagner PD, Breen EC (2010) Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. Am J Physiol Regul Integr Comp Physiol 299:R1059-R1067

Park H, Bhalla R, Saigal R, Radisic M, Watson N, Langer R, Vunjak-Novakovic G (2008) Effects of electrical stimulation in C2C12 muscle constructs. J Tissue Eng Regen Med 2:279-287

Parsons SA, Millay DP, Wilkins BJ, Bueno OF, Tsika GL, Neilson JR, Liberatore CM, Yutzey KE, Crabtree GR, Tsika RW, Molkentin JD (2004) Genetic loss of calcineurin block mechanical overload-induced skeletal muscle fibre type switching but not hypertrophy. J Biol Chem 279:26192-26200

Partridge T (2005) Versatility and commitment in muscle. J Physiol 562:646

Patti ME, Butte Aj, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci USA 100:8466-8471

Pedrotty DM, Koh J, Davis BH, Taylor DA, Wolf P, Niklasson LE (2005) Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation. Am J Physiol Heart Circ Physiol 288:H1620-H1626

Petroff OA, Prichard JW, Ogino T, Shulman RG (1988) Proton magnetic resonance spectroscopic studies of agonal carbohydrate metabolism in rabbit brain. Neurology 38:1569-1574

Pette D, Staron RS (2000) Myosin isoforms, muscle fibre types, and transitions. Microsc Res Tech 50:500-509

Pette D, Staron RS (2001) Transitions of muscle fibre phenotypic profiles. Histochem Cell Biol 115:359-372

Phillips SM (2009) Physiologic and molecular bases of muscle hypertrophy and atrophy: impact of resistance exercise on human skeletal muscle (protein and exercise dose effects). Appl Physiol Nutr Metab 34:403-410

Phillips SM, Tipton KD, Aarsland A, Wolf SE, Wolfe RR (1997) Mixed muscle protein synthesis and breakdown after resistance exercise in humans. Am J Physiol 273:E99-E107

Philp A, Hamilton DL, Baar K (2010) Signals mediating skeletal muscle remodelling by resistance exercise: PI3-kinase independent activation of mTORC1. J Appl Physiol doi:10.1152/japplphysiol.00941.2010

Pierce JR, Clark BC, Ploutz-Snyder LL, Kanaley JA (2006) Growth hormone and muscle function responses to skeletal muscle ischaemia. J Appl Physiol 101:1588-1595

Pilegaard H, Ordway GA, Saltin B, Neufer PD (2000) Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. Am J Physiol Endocrinol Metab 279:E806-E814

Pilegaard H, Osada T, Andersen LT, Helge JW, Saltin B, Neufer PD (2005) Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. Metabolism 54:1048-1055

Pilegaard H, Saltin B, Neufer PD (2003) Exercise induces transient transcriptional activation of the PGC-1 alpha gene in human skeletal muscle. J Physiol 546:851-858

Poehlman ET, Dvorak RV, DeNino WF, Brochu M, Ades PA (2000) Effects of resistance training and endurance training on insulin sensitivity in nonobese, young women: a controlled randomised trail. J Clin Endocrinol Metab 85:2463-2468

Poortmans JR, Saerens P, Edelman R, Vertongen F, Dorchy H (1986) Influence of the degree of metabolic control on physical fitness in type 1 diabetic adolescents. Int J Sports Med 7:232-235

Porter MM, Koolage CW, Lexell J (2002) Biopsy sampling requirements for the estimation of muscle capillarisation. Muscle Nerve 26:546-548

Prior BM, Yang HT, Terjung RL (2004) What makes vessels grow with exercise training? J Appl Physiol 97:1119-1128

Puigserver P, Rhee J, Lin J, Wu Z, Yoon J, Zhang C, Krauss S, Mootha V, Lowell B, Spiegelman BM (2001) Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPAR gamma coactivator-1. Mol Cell 8:971-982

Puigserver P, Spiegelman BM (2003) Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr Rev 24:78-90

Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 92:829-839

Putman CT, Dusterhoft S, Pette D (2000) Satellite cell proliferation in low frequency-stimulated fast muscle of hypothyroid rat. Am J Physiol Cell Physiol 279:C682-C690

Reeves GV, Kraemer RR, Hollander DB, Clavier J, Thomas C, Francois M, Castracane VD (2006) Comparison of hormone responses following light resistance exercise with partial vascular occlusion and moderately difficult resistance exercise without occlusion. J Appl Physiol 101:1616-1622

Regensteiner JH, Bauer TA, Reusch JE, Quaife RA, Chen MY, Smith SC, Miller TM, Groves BM, Wolfel EE (2009) Cardiac dysfunction during exercise in uncomplicated type 2 diabetes. Med Sci Sports Exerc 41:977-984

Rennie MJ, Wackerhage H, Spangenburg EE, Booth FW (2004) Control of the size of the human muscle mass. Annu Rev Physiol 66:799-828

Ribisl PM, Lang W, Jaramillo SA, Jakicic JM, Stewart KJ, Bahnson J, Bright R, Curtis JF, Crow RS, Soberman JE; Look AHEAD Research Group (2007) Exercise capacity and cardiovascular/metabolic characteristics of overweight and obese individuals with type 2 diabetes: the Look AHEAD clinical trial. Diabetes Care 30:2679-2684

Richardson RS, Wagner H, Mudaliar SR, Henry R, Noyszewski EA, Wagner PD (1999) Human VEGF gene expression in skeletal muscle: effect of acute normoxic and hypoxic exercise. Am J Physiol 277:H2247-H2252

Richter EA, Ruderman NB (2009) AMPK and the biochemistry of exercise: implications for human health and disease. Biochem J 418:261-275

Ristow M, Zarse K, Oberbach A, Kloting N, Birringer M, Kiehntopf M, Stumvoll M, Kahn CR, Blueher M (2009) Antioxidants prevent health-promoting effects of physical exercise in humans. Proc Natl Acad Sci USA 106:8665-8670

Rittweger J (2010) Vibration as an exercise modality: how it may work, and what its potential might be. Eur J Appl Physiol 108:877-904

Roca J, Agusti AG, Alonso A, Poole DC, Viegas JA, Rodriguez-Roisin R, Ferrer A, Wagner PD (1992) Effects of training on muscle O2 transport at VO2max. J Appl Physiol 73:1067-1076

Rockl KS, Witczak CA, Goodyear LJ (2008) Signalling mechanisms in skeletal muscle: acute responses and chronic adaptations to exercise. IUBMB Life 60:145-153

Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P (2005) Nutrient control of glucose homeostasis through a complex of PGC-1 alpha and SIRT1. Nature 434:113-118

Romanello V, Sandri M (2010) Mitochondrial biogenesis and fragmentation as regulators of muscle protein degradation. Curr Hypertens Rep 12:433-439

Russell AP, Feilchenfeldt J, Schreiber S, Praz M, Crettenand A, Gobelet C, Meier CA, Bell DR, Kralli A, Giacobino JP, Deriaz O (2003) Endurance training in humans leads to fibre type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. Diabetes 52:2874-2881

Sahlin K (1978) Intracellular pH and energy-metabolism in skeletal muscle of man, with special reference to exercise. Acta Physiol Scand 455:1-56

Sala E, Roca J, Marrades RM, Alonso J, Gonzalez De Suso JM, Moreno A, Barbera JA, Nadal J, De Jover L, Rodriguez-Roisin R, Wagner PD (1999) Effects of endurance training on skeletal muscle bioenergetics in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 159:1726-1734

Saltin, B, Blomqvist G, Mitchell JH, Johnson RL, Wildenthal K, Chapman CB (1968) Response to exercise after bed rest and after training. Circulation 38:S1-S78

Saltin B, Henriksson J, Nygaard E, Andersen P, Jansson E (1977) Fibre types and metabolic potentials of skeletal muscles in sedentary man and endurance runners. Ann N Y Acad Sci 301:3-29

Saltin B, Kiens B, Savard G, Pedersen PK (1986) Role of haemoglobin and capillarisation for oxygen delivery and extraction in muscular exercise. Acta Physiol Scand 556:21-32

Santos JM, Ribeiro SB, Gaya AR, Appell HJ, Duarte JA (2008) Skeletal muscle pathways of contraction-enhanced glucose uptake. Int J Sports Med 29:785-794

Scarpulla RC (2002) Nuclear activators and coactivators in mammalian mitochondrial biogenesis. Biochim Biophys Acta 1576:1-14

Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev 88:611-638

Schiaffino S, Sandri M, Murgia M (2007) Activity-dependent signalling pathways controlling muscle diversity and plasticity. Physiology 22:269-278

Schrauwen-Hinderling VB, Roden M, Kooi ME, Hesselink MK, Schrauwen P (2007) Muscular mitochondrial dysfunction and type 2 diabetes mellitus. Curr Opin Nutr Metab Care 10:698-703

Schreiber SN, Emter R, Hock MB, Knutti D, Cardenas J, Podvinec M, Oakeley EJ, Kralli A (2004) The estrogen-related receptor alpha (ERR alpha) functions in PPAR gamma coactivator 1 alpha (PGC-1 alpha)-induced mitochondrial biogenesis. Proc Natl Acad Sci USA 101:6472-6477

Schreiber SN, Knutti D, Brogli K, Uhlmann T, Kralli A (2003) The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor alpha (ERR alpha). J Biol Chem 278:9013-9018

Serres I, Gautier V, Varray A, Prefaut C (1998) Impaired skeletal muscle endurance related to physical inactivity and altered lung function in COPD patients. Chest 113:900-905

Seynnes OR, de Boer M, Narici MV (2007) Early skeletal muscle hypertrophy and architectural changes in response to high-intensity resistance training. J Appl Physiol 102:368-373

Silveira LR, Pilegaard H, Kusuhara K, Curi R, Hellsten Y (2006) The contraction induced increase in gene expression of peroxisome proliferator-activated receptor (PPAR)-gamma coactivator 1 alpha (PGC-1 alpha), mitochondrial uncoupling protein 3 (UCP3) and hexokinase II (HKII) in primary rat skeletal muscle cells is dependent on reactive oxygen species. Biochim Biophys Acta 1763:969-976

Slotboom J, Boesch C, Kreis R (1998) Versatile frequency domain fitting using time domain models and prior knowledge. Magn Reson Med 39:899-911

Smerdu V, Karsch-Mizrachi I, Campione M, Leinwand L, Schiaffino S (1994) Type IIx myosin heavy chain transcripts are expressed in type IIb fibres of human skeletal muscle. Am J Physiol 267:C1723-C1728

Spangenburg EE, Booth FW (2003) Molecular regulation of individual skeletal muscle fibre types. Acta Physiol Scand 178:413-424

St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jaeger S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM (2006) Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell 127:397-408

St-Pierre J, Lin J, Krauss S, Tarr PT, Yang R, Newgard CB, Spiegelman BM (2003) Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1 alpha and 1 beta (PGC-1 alpha and PGC-1 beta) in muscle cells. J Biol Chem 278:26597-26603

Staron RS (1997) Human skeletal muscle fibre types: delineation, development, and distribution. Can J Appl Physiol 22:307-327

Staron RS, Karapondo DL, Kraemer WJ, Fry AC, Gordon SE, Falkel JE, Hagerman FC, Hikida RS (1994) Skeletal muscle adaptations during early phase of heavy resistance training in men and women. J Appl Physiol 76:1247-1255

Staron RS, Leonardi MJ, Karapondo DL, Malicky ES, Falkel JE, Hagerman FC, Hikida RS (1991) Strength and skeletal muscle adaptations in heavy resistance-trained women after detraining and retraining. J Appl Physiol 70:631-640

Stern-Straeter J, Bach AD, Stangenberg L, Foerster VT, Horch RE, Stark GB, Beier JP (2005) Impact of electrical stimulation on three-dimensional myoblast cultures: a real-time RT-PCR study. J Cell Mol Med 9:883-892

Suhr F, Brixius K, de Marees M, Boelck B, Kleinoeder H, Achtzehn S, Bloch, Mester J (2007) Effects of short-term vibration and hypoxia during high-intensity cycling exercise on circulating levels of angiogenic regulators in humans. J Appl Physiol 103:474-483

Sundberg CJ (1994) Exercise and training during graded leg ischaemia in healthy man with special reference to effects on skeletal muscle. Acta Physiol Scand 615:S1-S50

Swoap SJ, Hunter RB, Stevenson EJ, Felton HM, Kansagra NV, Lang JM, Esser KA, Kandarian SC (2000) The calcineurin-NFAT pathway and muscle fibre-type gene expression. Am J Physiol Cell Physiol 279:C915-C924

Takarada Y, Sato Y, Ishii N (2002) Effects of resistance exercise combined with vascular occlusion on muscle function in athletes. Eur J Appl Physiol 86:308-314

Takarada Y, Takazawa H, Sato Y, Takebayashi S, Tanaka Y, Ishii N (2000) Effects of resistance exercise combined with moderate vascular occlusion on muscular function in humans. J Appl Physiol 88:2097-2106

Taylor EB, Lamb JD, Hurst RW, Chesser DG, Ellingson WJ, Greenwood LJ, Porter BB, Herway ST, Winder WW (2005) Endurance training increases skeletal muscle LKB1 and PGC-1 alpha protein abundance: effects of time and intensity. Am J Physiol Endocrinol Metab 289:E960-E968

Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, Tabata I (2002) Effects of low- intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochem Biophys Res Commun 296:350-354

Tesch PA (1988) Skeletal muscle adaptations consequent to long-term heavy resistance exercise. Med Sci Sports Exerc 20:S132-S134

Tesch PA, Ekberg A, Lindquist DM, Trieschmann JT (2004) Muscle hypertrophy following 5-week resistance training using a non-gravity-dependent exercise system. Acta Physiol Scand 180:89-98

Thelen MH, Simonides WS, van Hardeveld C (1997) Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent transcription of the fast-type sarcoplasmic-reticulum Ca^{2+}-ATPase gene. Biochem J 321:845-848

Thomas PE, Ranatunga KW (1993) Factors affecting muscle fibre transformation in cross-reinnervated muscle. Muscle Nerve 16:193-199

Timmons JA, Norrbom J, Scheele C, Thonberg H, Wahlestedt C, Tesch P (2006) Expression profiling following local muscle inactivity in humans provides new perspective on diabetes-related genes. Genomics 87:165-172

Toigo M, Boutellier U (2006) New fundamental resistance exercise determinants of molecular and cellular muscle adaptations. Eur J Appl Physiol 97:643-663

Unwin N, Shaw J, Zimmet P, Alberti KG (2002) Impaired glucose tolerance and impaired fasting glycaemia: the current status on definition and intervention. Diabet Med 19:708-723

Veves A, Saouaf R, Donaghue VM, Mullooly CA, Kistler JA, Giurini JM, Horton ES, Fielding RA (1997) Aerobic exercise capacity remains normal despite impaired endothelial function in the micro- and macrocirculation of physically active IDDM patients. Diabetes 46:1846-1852

Vina J, Gimeno A, Sastre J, Desco C, Asensi M, Pallardo FV, Cuesta A, Ferrero JA, Terada LS, Repine JE (2000) Mechanism of free radical production in exhaustive execise in humans and rats; role of xanthine oxidase and protection by allopurinol. IUBMB Life 49:539-544

Vissing K, Andersen JL, Schjerling P (2005) Are exercise-induced genes induced by exercise? FASEB J 19:94-96

Vollaard NB, Constantin-Teodosiu D, Fredriksson K, Rooyackers O, Jansson E, Greenhaff PL, Timmons JA, Sundberg CJ (2009) Systemic analysis of adaptations in aerobic capacity and submaximal energy metabolism provides a unique insight into determinants of human aerobic performance. J Appl Physiol 106:1479-1486

Wallberg-Henriksson H, Gunnarsson R, Henriksson J, Ostman J, Wahren J (1984) Influence of physical training on formation of muscle capillaries in type 1 diabetes. Diabetes 33:851-857

Walti MK, Walczyk T, Zimmermann MB, Fortunato G, Weber M, Spinas GA, Hurrell RF (2006) Urinary excretion of an intravenous ^{26}Mg dose as an indicator of marginal magnesium deficiency in adults. Eur J Clin Nutr 60:147-154

Wang N, Hikida RS, Staron RS, Simoneau JA (1993) Muscle fibre types of women after resistance training – quantitative ultrastructure and enzyme activity. Pflugers Arch 424:494-502

Wenger RH (2000) Mammalian oxygen sensing, signalling and gene regulation. J Exp Biol 203:1253-1263

Wildman RP, Muntner P, Reynolds K, McGinn AP, Rajpathak S, Wylie-Rosett J, Sowers MR (2008) The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: prevalence and correlates of two phenotypes among the US population (NHANES 1999-2004). Arch Intern Med 168:1617-1624

Wilkin TJ (2001) The accelerator hypothesis: weight gain as the missing link between type 1 and type 2 diabetes. Diabetologia 44:914-922

Williams RS, Neufer PD (1996) Regulation of gene expression in skeletal muscle by contractile activity. In: The handbook of physiology. Exercise: regulation and integration of multiple systems. Oxford University Press, New York, USA, pp 1124-1150

Wilson JR, Martin JL, Schwartz D, Ferraro N (1984) Exercise intolerance in patients with chronic heart failure: role of impaired nutritive flow to skeletal muscle. Circulation 69:1079-1087

Winter EM, Fowler N (2009) Exercise defined and quantified according to the Système International d'Unités. J Sports Sci 27:447-460

Wright DC, Han DH, Garcia-Roves PM, Geiger PC, Jones TE, Holloszy JO (2007) Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1 alpha expression. J Biol Chem 282:194-199

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98:115-124

Yamaguchi T, Suzuki T, Arai H, Tanabe S, Atomi Y (2010) Continuous mild heat stress induces differentiation of mammalian myoblasts, shifting fibre-type from fast to slow. Am J Physiol Cell Physiol 298:C140-C148

Yan Z, Okutsu M, Akhtar YN, Lira VA (2010) Regulation of exercise-induced fibre type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle. J Appl Physiol doi:10.1152/japplphysiol.00993.2010

Contributions to the publications and manuscripts

This thesis consists of the following original publication and manuscripts and I contributed to it as indicated:

Study 1 (Chapter 2):
Item F*, Heinzer-Schweizer S*, Wyss M, Fontana P, Lehmann R, Henning A, Weber M, Boesiger P, Boutellier U, and Toigo M (2010) Oxidative capacity is affected by glycaemic status in young untrained women with type 1 diabetes but is not impaired relative to healthy untrained women. Am J Physiol Regul Integr Comp Physiol
In revision (*Contributed equally to this work)

- Everything, except ^{31}P-MRS measurements, graded cycling exercise and constant load tests, and blood analyses

Study 2 (Chapter 3):
Burch N, Arnold AS, Item F, Summermatter S, Brochmann Santana Santos G, Christie M, Boutellier U, Toigo M, and Handschin C (2010) Electric pulse stimulation of cultured murine muscle cells reproduces gene expression changes of trained mouse muscle. PLoS One 5:e10970

- Method establishment: Electric pulse stimulation (EPS) system
- Substantial support of Burch N (MSc student) with cell culture work

Study 3 (Chapter 4):
Toigo M, Item F, Denkinger J, Fontana P, Weber M, and Boutellier U (2010) Adaptations to simultaneous vibration and resistance exercise with vascular occlusion. *Submitted*

- Everything, except the graded cycling exercise and constant load tests
- Assisted by a MSc-student

Study 4 (Chapter 5):
Item F, Nocito A, Thoeny S, Baechler T, Boutellier U, Wenger RH, and Toigo M (2011) Modified resistance exercise increases PGC-1α and VEGF mRNA abundances. *In preparation*

- Everything
- Assisted by a MSc-student

Additional manuscript (not included in this thesis):

Annaheim S, Anliker L, Item F, Boutellier U, and Toigo M (2011) Effects of prolonged cycling exercise on muscular metabolism and economically optimal cadence related to muscle fibre type composition. *In preparation*

- Histological analyses of skeletal muscle tissue

Curriculum vitae

Personal	ITEM Flurin Industriestrasse 33, 8610 Uster ZH, Switzerland Born June 15th, 1981 in Chur GR, Switzerland citizen of Marmorera GR, Switzerland	
Education	since 2007	PhD student in Exercise Physiology, Institute of Physiology, University of Zurich, and Institute of Human Movement Sciences, ETH Zurich, Switzerland
	2002-2007	Master of Science in Human Movement Sciences, Major in Exercise Physiology, ETH Zurich, Switzerland
	1996-2001	Gymnasium, Typus C, Chur, Switzerland
	1988-1996	Primar- and Sekundarschule, Rhäzüns, Switzerland
Oral presentations	2010	Seminar lecture, Institute of Physiology, University of Zurich, Switzerland
	2009	5th ZIHP Symposium, University Hospital, Zurich, Switzerland

Scientific publications

Burch N, Arnold AS, Item F, Summermatter S, Brochmann Santana Santos G, Christine M, Boutellier U, Toigo M, Handschin C (2010) Electric pulse stimulation of cultured murine muscle cells reproduces gene expression changes of trained mouse muscle. PloS One 5:e10970

Niessen M, Jaschinski F, Item F, McNamara MP, Spinas GA, Trueb T (2007) Insulin receptor substrates 1 and 2 but not Shc can activate the insulin receptor independent of insulin and induce proliferation in CHO-IR cells. Exp Cell Res 313:805-815

Acknowledgements

Herewith, I would like to thank everyone, who contributed to the studies involved in my doctoral thesis and/or supported me during the last years:

Dr. Marco Toigo, Prof. Dr. Roland H. Wenger, and Prof. Dr. med. Urs Boutellier for giving me scientific support during the last years.

Prof. Dr. Christoph Handschin and Dr. Gieri Camenisch, who took place in my doctoral thesis committee.

All study participants for their effort and enthusiasm.

The members of the “Exercise Physiology” and “Cellular Oxygen Physiology” groups and in this regard, especially my colleagues from Y23 M 48.

My parents, Dorli and Gion for their support throughout all my education.

My wife, Julia for her patience, solid support and encouragement.